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Wiese, Dr. Conrad
Wilhelm, Dr. Hazel S.
Wilson, Mr. and Mrs. T. Hastings
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Woodwell, Dr. and Mrs. George M.
Yntema, Mrs. Chester L.
Zinn, Dr. and Mrs. Donald J.
Zipf, Dr. Elizabeth
Zwilling, Mrs. Edgar

III. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We. Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 11, 1978)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of 4 years. Eligibility for re-election shall be in accordance with the content of Article VIII(F) as applied to corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII(B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as ex officio members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification.

VI. REPORT OF THE DIRECTOR

Those who do concentrated work discover quickly a useful device for resting the eyes and refreshing the power of attention: it is to look away from the job from time to time: to focus briefly at infinity. Just so I alternate now between writing this report, *i.e.*, representing the year just past at the MBL without *cataloging* it (which is the purpose of the remainder of this volume), and correcting galleys for a supplementary volume of *The Biological Bulletin,* to be published in June of 1985.

Paradoxically, the *close* work here is this Director's Report, with its problem of representation, while the infinite focus is on the small-print page proofs, which deal with the MBL's ninety-seven year history. They are the printed versions of papers read at a meeting held on the Island of Ischia in October, 1984, the purpose of which was to examine the scientific and political histories of the MBL and the Stazione Zoologica in Naples.

If it is not overdone, this switching back and forth refreshes the powers of concentration, almost as does looking out the window over the blue expanse of Great Harbor when one has been counting autoradiographic silver grains, or preparing a squid axon. However routine or exciting, confident or threatening were individual months and years of the MBL's history, the whole sweep of it is a smooth curve that engages the eye. It is a rest from the ups and downs of a single year in retrospect.

In this Centennial year minus four (minus three at the time of publication) much more will be said about the smooth curve. Planning for 1988 will get underway; inevitably, plans for celebrations will be considered in the light of program plans for the MBL of today and tomorrow. I trust that most members of the Corporation will involve themselves. There is a great deal to be learned, not merely about MBL science of the past, and the institutional processes that generated it, but also about MBL science of the future, and what initiatives we ought to take, matching courage with the founders, in order to insure continued excellence.

MBL science

Literalists sometimes question my use of that adjective, "MBL." "Science," they say, "is science." "The fact that it's done at the MBL, rather than at some *Technische Hochschule,* doesn't alter the content. If it contributes to the understanding of nature, then it comes from, and belongs to, the whole of biology." I cannot disagree. But by "MBL science" I mean neither the trivial localization of hands and equipment, nor to suggest that scientific work done here is somehow distinct from the whole body of exact knowledge about the world. I mean to imply, rather, that there are particular conditions for scientific advance that occur regularly at this Laboratory, and not so regularly elsewhere. I mean that those conditions are responsible for the extraordinary impact this Laboratory and its people have had upon biomedical science, despite our small size, small wealth, and too small a public visibility. These implications were the basis of our choice of the title "MBL Science" for the magazine that is the most recent effort of our Public Information office.

A case in point lies among the papers of today's mail, which litter my desk and are the despair of the office staff. This is a clipping from the *London Times,* one of the press reports on recent discoveries concerning fast axonal transport. No one who has observed living things under a good microscope fails to be impressed by the rapid and directed motions made by subcellular organelles. That such transport processes are critical for normal function is most obvious in the neuron, where the products of biosynthesis in the cell body, including transmitter substances, must traverse enormous distances to reach their sites of action at the axon terminals. The fast components of organellar transport are very fast indeed: forty centimeters per day is not uncommon.

MBL scientists, like others around the world, have been observing slow and fast axonal transport for some years, and contributing to its accurate description. But the sudden advances to which the *London Times* refers would not have been so sudden had the contributors not been MBL people, summer and year-round, and had they not participated in the unique sharing of technology, of ideas, and of criticism that *is* the MBL. This sharing breaks down the barriers between laboratories, between teaching and research, between disciplines. The *Times* article identifies the investigators only as "of Woods Hole, Massachusetts." Other popular accounts identify them only with NIH, or with NINCDS. But they, and other contributors not mentioned in these reports, are in fact "of the Marine Biological Laboratory, Woods Hole, Massachusetts." Their abruptly newsworthy achievements represent well what is meant by "MBL science."

The news is about the clear identification of *microtubules* as the physical tracks over which mitochondria, vesicles of various kinds and dimensions, and probably macromolecules traverse the vast distances that separate the perinuclear cytoplasm from the axon terminals. The identification itself is important; but perhaps more important is the new information, emerging from this work, on constancy of transport speed in extruded axoplasm, on the transport of model (artificial) organelles, and on the translocator protein(s) necessary for transport to occur. There is reason to hope that the chemistry of fast axonal transport will soon be worked out, as was the chemistry of muscle contraction, because the new data place strict limits on the components, energetics, and kinetics of the process.

This work is "MBL science" for more reasons than the fact that it was done at the MBL, and on the giant axon of the Woods Hole squid. There is, first, the observational background at the light-microscope level. That required the often parallel, sometimes divergent development of video differential interference contrast microscopy in two laboratories—those of R. D. Allen, a summer investigator at the MBL, and S. Inoué, a member of the year-round scientific staff. This technology, and the long-time interest of both laboratories in particulate transport processes, were and are freely available here. Success required the unique ultrastructural expertise of T. Reese, an NIH scientist who is posted year-round at the MBL, and who has been co-Director of the MBL's Neurobiology course. Collaborating in the venture were several other MBL investigators, some of whom were attracted to the work while serving as fellows or as faculty of the course. And it required the community-wide interest in, and knowledge of, the chemistry of microtubules and other cytoskeletal elements, as represented in the work of such scientists as R. E. Stephens and the current leadership of the Physiology course.

The most recent outcome of all those interacting skills is a group of scientific papers published by Reese and his colleagues R. D. Vale, B. J. Schnapp, and M. P. Sheetz. These papers prove, by an elegant method of observing with the electron microscope (hence at appropriately high resolution) precisely the same particles and tracks that were observed functioning earlier, at lower resolution, on the screen of the video-microscope.

To set the story in such a context is not to deny credit to the authors of these last and noteworthy papers. It is, rather, to suggest how critical for the ability to do the right experiments at the right time, by the best methods, is the interactive style of MBL science. MBL science has evolved as an integrated whole: the courses; the visiting investigators who are not (for the moment) teaching; the year-round scientific staff, whether on location or permanently in the MBL's employ. Ten years into the jigsaw-puzzle of building a year-round research program to match the quality of, and relate closely to, the summer programs, year-round science is already integral to the life of the MBL. Ninety-seven years into the curious plan of eclecticism and change that characterizes the instructional program, it remains uniquely productive of important new science as well as of scientists; and it is just as closely intertwined with research as when Jacques Loeb first lectured on tropisms, "irritability," and the universality of ion effects on "protoplasm."

MBL science *is* definable: and it is in very good condition. There are a dozen other 1984-85 stories I might have told here, in addition to, or instead of, the one about fast axonal transport (although that one may well end as having received the most publicity). All those stories will be published and will have their impact upon biology and human health. Some of them will become known to the lay public. A few of them will be associated in the public mind with Woods Hole; and fewer yet with this particular one of the Woods Hole institutions.

We are doing much better than in the past at public relations, and therefore at fund-raising, but I do not expect "MBL" to become a household word. No matter: MBL science exists and its value is known where it counts most: among biomedical scientists. In every year since I began writing these reports, it has, by the most objective measures, grown stronger and better-integrated. Were we able to make a report to C. O. Whitman, whose idea the MBL really was, it would be a proud one. We *would* find it necessary, of course, to speak of urgent needs; but he would be entirely familiar with that.

Eclecticism and change

The description of a work, or a body of works, as "eclectic" tends to be pejorative, particularly in times of uncertain values. Witness today's art criticism in the context of the art market: nobody, not even the artist, has any reliable standard by which to judge the value of work. In such a case, the value is set by what the highest bidder will pay. But high bidders value novelty above all else. An artist, whose approach is said to be eclectic, therefore, doesn't make it with buyers and critics, whose sole concern—for different reasons—is novelty. But I used "eclectic," in describing the MBL courses, as praise, and in its original sense, which is that of selection: selection of what appears at any time to be best among all possible styles, methods, and approaches. Eclecticism in the training of scientists is the *only* way to go. And by that token there must be change. What is, or appears to be, the best science to teach in 1980 cannot be, or appear to be, best in 1985. Not in a time when the foundations of biomedical science shake a little with every other issue of *Nature*, or *Science*, or *Cell*.

Eclecticism and change were more evident than usual during the past year, for there were four new directorates of summer courses to be recommended by search committees, to be cajoled, and to be organized. It is a pleasure to report that the process, which is surely no less complex than finding a new Department Chairman in a university, has succeeded as MBL tradition demands, despite the rare coincidence of four changes in one year.

John Hildebrand and Tom Reese retired from co-directorship of the Neurobiology course after a five-year tenure that matched in quality and conscientiousness the leadership of their predecessor, Edward Kravitz. They are replaced by Arthur Karlin, of Columbia University, whose planned changes incorporate what is new at both ends of the course's spectrum of topics: the biochemistry and molecular biology of receptors and channels, and the biophysics of electrogenesis. Ron Hoy and Eduardo Macagno completed their successful co-directorship of the Neural Systems and Behavior course, and will be replaced by Darcy Kelley, also of Columbia, who will have assistance in directing the new version from Tom Carew of Yale. Harlyn Halvorson stepped down from his energetic leadership of the Microbial Ecology course, to be succeeded by Ralph Wolfe and Peter Greenberg (Illinois and Cornell, respectively). Their plan for the new version, which has been renamed "Microbiology: Molecular Aspects of Cellular Diversity," includes changes in the method of laboratory instruction and in the purposes of the program. The new thrust will be intensive laboratory training in the exploitation of microbial diversity. And finally, John David, founding director of the Biology of Parasitism course, has yielded, after a standard-setting five-year run of worldwide visibility, to Paul Englund of Johns Hopkins, assisted by Alan Sher of NIH. There is now reason to hope that a distinguished program in cellular and molecular plant biology can be initiated in 1986.

I do not mean it as self-congratulation for the MBL, but rather as honest wonder, when I say that this small place, whose name the newspapers seem never to get right (or that they ignore), accomplishes something extraordinary in every such change. To have replaced so distinguished a set of chief instructors with a new set equally strong, to have met their personal, technical, and organizational needs, and to have satisfied them on the benefits of the sacrifices they make in order to teach here, measures the true stature of this Laboratory better than any citations in the press. "The job is its own reward" is a cliché; but teaching at the MBL is the exception: it *is* its own reward, as any retired faculty member will testify. Still, I wish that we had some concrete process, more than "thank you," by which the MBL community might make known its gratitude to retiring faculty.

Change: operating departments

In no other branch of MBL functions is the need for change, as that is driven by research and teaching, so evident as within the operating Departments. However carefully planning and budgeting are done, the responsibility of Department Heads and staff is to respond promptly to program needs. Especially for the summer programs, this can decide between work accomplished and the opposite. When, as at the MBL, programs are opportunistic (in the positive sense of that word), the unforeseen must somehow be foreseen. The record of our Departments is far better than that of their equivalents in universities. This is not because we have greater financial leeway or are notably more ingenious, but because it is a real tradition here, rather than a token statement, that MBL employees (including the Director) *are here to help investigators and faculty*, rather than the reverse. I will cite just a few examples of operations responsiveness this past year.

Within Research Services, whose normal responsibilities and budgetary problems are better understood by the scientists than are those of other Departments, the following special initiatives had to be taken within the year: arranging for compliance in detail with newly-enacted "Right to Know" legislation, which calls for training programs, special record keeping, and new emergency measures, all concerned with hazardous materials. Organization of an Emergency Response Team, including persons qualified to administer cardiopulmonary resuscitation. Special arrangements for legal disposal of research animal carcasses. Reorganization of ordering and delivery systems for chemicals. New arrangements for collaboration with the Grants and Educational

Services Office on managing purchases, loans, and the safe, timely installation of equipment for the instructional program.

The Buildings and Grounds Department carried out its large program of routine maintenance and repairs, but it also carried out or directed the following: completion of new smoke and fire alarm systems in all MBL structures; new roofs on all Devil's Lane cottages; insulation, weather-stripping, and painting of extensive areas in Lillie and Loeb; construction of standard plywood boxes for storage of investigator equipment; many emergency or near-emergency plumbing jobs; gutters for the Candle House roof; and a start on rehabilitation of the fume-hood exhaust systems in the Environmental Sciences Center.

The Marine Resources Department designed and placed in operation a pilot recirculating chilled seawater system. Increased monitoring of species that are heavily pressured by commercial fishing or by rising investigator demand was instituted. These species include, among others, squid, goosefish, *Limulus, Solemya, Chaetopterus,* and *Microciona.* Special equipment was created to allow a distinguished investigator, here on sabbatical leave during the winter, to maintain juvenile lobsters (for developmental neurobiology) in warm water. And in response to difficulties caused by rising demand for finfish and an inadequate building (our historic but grossly obsolete Supply House), plans were drawn up, in collaboration with the Laboratory of Marine Animal Health (Dr. L. Leibovitz in charge), for expansion of dock space, the construction and installation of large-volume holding tanks, and new experimental work on finfish maintenance. This program is expected to be operational by the summer of 1985.

The Public Information office continued its successful effort of upgrading MBL publications as to frequency, content, and graphic design. An entirely new periodical magazine, *MBL Science,* has replaced the old *MBL Newsletter.* It has been received with unusual enthusiasm, not just within the MBL community, but outside it as well. The initiative of offering daily tours of the MBL during the summer succeeded almost too well: it required a rapid response. Within a week of the program's birth, tours were subscribed to capacity nearly every day. By the end of the 1984 summer, nearly 500 people had participated. This could not have been accomplished without the generous volunteer work of such MBL community members as Betsy Bang, Sears Crowell, Stubby and Julie Rankin, Margery Taylor, and Donald Zinn. A handsome exhibit, "Science and the Squid," graced the Lillie lobby and brought congratulations, not only to the Public Information office, but to the writer of this Report from dozens of visitors, especially to the Friday Evening Lectures.

Eclecticism: the Board Trustees

Positively eclectic, like the system of design and leadership of MBL courses, is the selection and election of our Board Trustees. Their importance to the Laboratory has grown steadily the past five years, and I expect it so to continue. Eclectic they are if the description refers to their qualifications and personal histories. The range is from distinguished scientists, only recently recruited to management or administrative roles of international significance, to bankers, investment specialists, and economists, equally distinguished in their professions. All our Trustees are volunteers, as trustees must properly be. But the MBL's scientist-trustees play a role that is much closer to that of the Board of Directors of a company than it is to an academic Board of Trustees.

For the MBL their role, too, has been and will continue to be vital. But as the Laboratory's needs, international reach, and requirements for public accountability have risen, so has its need for Trusteeship in the currently understood sense: the task of representing an institution to the public, and of representing, within an institution, the views and requirements of the public. I have written elsewhere, plagiarizing statements of my former boss, President Robert Sproull of Rochester, that Trustees must balance the present against the future. That is overwhelmingly the most important responsibility of the MBL's Board Trustees; and I am delighted to observe that they have been taking it more seriously, and executing it more effectively, each year. The task of integrating these accomplished men and women into the *working* life of the MBL remains, and I hope that the Corporation as a whole, as well as those of us in the Administration, can increase our efforts in that regard. It is the very least we can do for the Board Trustees, by way of thanks.

Needs

I noted earlier that the MBL's needs of today would not surprise C. O. Whitman, were he alive: he would understand perfectly that the MBL must, if it is to survive as a headlight, rather than a taillight, of biology, practice and teach the most advanced biology, no matter where it leads, no matter what biological materials are used for experimentation, and no matter how much it costs. It was Whitman who once expostulated "Good Lord, what is money for?"

The MBL has done remarkably well, upon the basis of insignificant endowment resources and a nearly impossible administrative work load for those who look after it, in holding to Whitman's principle of universality and highest quality. But as I have noted in earlier Reports, the game is much harder than in the past—harder, even, than it was in 1978. To play it effectively, we must, somehow, meet its demands, and we must meet them soon. Some of them should be met by the Centennial year. I refer to several urgencies of which most Corporation members are already aware:

A new Marine Resources Center, without which, sooner rather than later, the provision of research specimens will fall into decline, research on their laboratory culture and genetic definition will stop, and the year-round scientific program in general will be brought into disarray.

New housing, especially for students, younger investigators, and families, without which the future will be sacrificed to the past because these members of our community cannot afford commercial summer housing in a resort area.

Education endowment, needed because, despite generous and encouraging private grants in support of MBL instruction, only significant income in perpetuity will allow us the eclecticism and change in teaching that is our hallmark. I need perhaps not enlarge here upon the truth that, with the decline of advanced laboratory instruction in universities, the MBL training programs are ever more indispensable to the maintenance of national manpower in biomedical science.

Strengthening of administrative manpower and skill, to insure that the sealing-wax and shoestring that hold together our far-flung enterprise, however ingeniously applied and however unselfishly some have given up energy and even other careers to it, are replaced by proper sealants and fasteners, suited to a space-age structure.

I hope that the Corporation will join me, as the Centennial approaches, in rededication to meeting those needs. There is no reason why they can't be met: the thing is feasible; it is important for science; and it is significant for the culture. The funds are available: a tiny fraction of what is given each year for religion and pet care would do the trick. We need only the active help, the ideas, and a little of the time of everyone who knows the MBL, cares about it, and benefits from its existence.

VII. REPORT OF THE TREASURER

Last fall, feeling greatly increased pressure from business obligations, I reluctantly asked the Chairman of our Board of Trustees to seek my successor as the MBL's Treasurer. Accordingly, at their February meeting, the Trustees elected Mr. David

Currier to succeed me as Treasurer. Mr. Currier recently retired from a senior management position in Boston's Shawmut Bank and now resides on Martha's Vineyard. In addition to a broad managerial and financial background, Mr. Currier brings to the MBL a special knowledge of real estate and its financing. His experience should prove invaluable as the MBL continues to review its options with respect to its real property holdings and its housing needs.

As I transfer my treasury-keeping duties to my successor, I think it appropriate to summarize here a few observations about the MBL's recent financial history and to comment on what appears to be in the offing.

Perhaps the thought will startle the reader, but my first reflection focuses on the fact that the MBL survived my tenure of office! Although this might be taken as an expression of amazement about my own capabilities (and there may be some justification for such wonderment), I mean only that my six years as Treasurer coincided with events that truly threatened the survival of all research centers dependent on federal grants and private gifts. The raging inflation of a few years ago slashed the purchasing power of resources already too limited. As this threat subsided, federal cutbacks in funding for research not only intensified the competition for federal money but also resulted in heavier pressure on private sources of funding. That the MBL survived these threats is not only a tribute to its fundamental strength but is also a very positive reflection on the fiscal leadership contributed by the MBL's full-time management.

I have commented in previous annual reports that adversity, while vexing to be sure, also has its benefits. Coping tends to strengthen. The necessity to deal with threats to its financial well-being has made the MBL stronger than before. I cite, for example, the installation of a new, automated accounting system in 1983 which greatly accelerated the production of management reports with which to control expenditures and to track financial performance against budgets. Among its many benefits, this system has significantly reduced receivables, thereby diminishing the extent to which the MBL is extending free "credit" to our investigators' home institutions.

The financial challenges of the recent past also prompted a review of overhead recovery. The MBL unquestionably has offered an unparalleled bargain in terms of the cost of doing research in our laboratories. Partly, this has been true because we have under-recovered legitimate overhead costs. A new overhead recovery system has been developed with input from many of the investigators who use our facilities, and now awaits final approval from the cognizant federal agency. Work at the MBL will still be a bargain, but at least we will be assured of greater recovery on all proper overhead items.

Financial white water also caused the MBL to review carefully the true cost of each of its major programs and the offsetting revenues. In this review, particular attention was directed to the MBL's instructional programs. Heretofore, it was widely believed that our educational efforts are self-sustaining. The facts tell us otherwise. Education is a major purpose for the MBL's existence, and so it is not reasonable to abandon these programs as a means to improve financial health. Instead, intensive efforts were directed at finding supplemental funding. I am happy to report that these efforts have been rewarding, and the gap between the costs of our educational program and revenues to support them has been significantly narrowed.

Although important steps have been taken to strengthen the MBL's financial vitality, there is still much to be done. One matter of some disappointment to me has been our inability to recognize the reality of depreciation. This year we are again able to report a modest surplus of income over expenses. Note however, as has always been true in our financial reporting, the results for the fiscal year are reported *before provision for depreciation*. Ideally, we should be using an annual depreciation charge to fund a reserve for the repair and replacement of our physical assets. Without such a reserve, we will continue to be faced with the need to seek gifts to finance remodeling and repairs.

A second disappointment has been our continuing dependence upon annual contributions and gifts for ordinary operating expenses. Increased fees, charges, and tuitions are probably not the answer, although these sources of income must continue to be adjusted for rising costs. Instead, we must look to the Second Century Campaign to add endowment and we must seek new sources of revenues. On the latter point, the possibility of increased winter utilization of our conference and housing facilities is being actively explored.

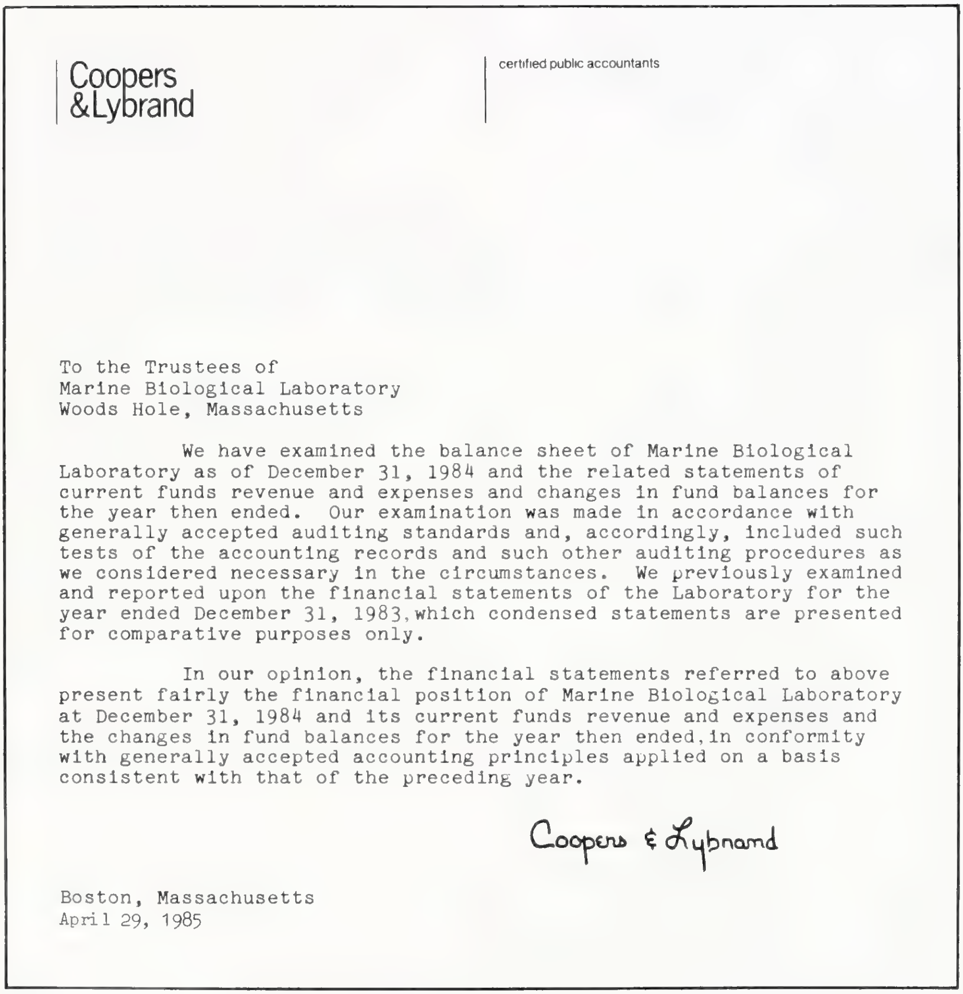
Mention of the Second Century Campaign brings me to my third disappointment, which has been the MBL's struggle to mount a broadly based, vigorous and imaginative development program. Much of the development work continues to center excessively on our Chairman and our President/Director. In contrast to universities, hospitals, and other institutions which can look to large numbers of beneficiaries to assist with fund raising, the MBL must depend on a much smaller cadre of solicitors. Moreover, many of the persons best able to speak for the MBL are also burdened with an obligation to find money for their own research or to assist their home institutions. In this situation, it is imperative that the presently vacant position of Development Director be filled as quickly as possible with a person highly skilled in making effective use of limited fund raising resources.

Looking ahead to the years immediately before us, the MBL must deal with several issues of significant financial import. We have dealt cautiously with our holdings of undeveloped real estate situated in a popular community where raw land commands premium prices. It has been tempting to consider the conversion of this unproductive asset into an income-producing asset, either through development or by sale and subsequent reinvestment. On the other hand, carrying costs for the land are low and its rate of appreciation in value has at least equaled, and probably exceeded, the rate of growth expected from securities investments. My personal inclination has been to treat our undeveloped real estate as a reserve against the possibility that financial threats even greater than those recently seen might jeopardize the MBL's ability to fulfill its purposes. However, the policy questions related to our real estate holdings are of substantial significance and should be revisited with regularity.

Intertwined with our real estate policy is the matter of housing. Clearly, steps must be taken to assure access to the MBL by young scientists and students. The rising cost and diminished availability of housing in the Woods Hole area will effectively preclude many young talents from taking advantage of the MBL's offerings unless we find practical solutions. The solutions will be expensive but necessary.

Lastly, I cite the Marine Resources Center as a project with subtle financial implications which may be overshadowed by the challenge of finding funds for its construction. As I became better acquainted with the MBL's history during my tenure as Treasurer, I developed an appreciation for what was required to make the MBL "work" as a viable research and educational center at various points in time. For today's needs, the proposed Marine Resources Center is a vital ingredient in the mix of capabilities and facilities required for continued viability. If the MBL doesn't "work" as an organic whole containing within it all the functionality needed by its users, it also doesn't "work" financially. In this sense, the Marine Resources Center is as important to your Treasurer as it is to MBL scientists.

I close my report with a word of appreciation to the many persons who have made my association with the MBL a stimulating, enjoyable experience. A special thanks goes to John Speer, Controller, who not only manages the MBL's financial affairs with great skill and dedication, but also has made the Treasurer look good.



Coopers
&Lybrand

certified public accountants

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1984 and the related statements of current funds revenue and expenses and changes in fund balances for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported upon the financial statements of the Laboratory for the year ended December 31, 1983, which condensed statements are presented for comparative purposes only.

In our opinion, the financial statements referred to above present fairly the financial position of Marine Biological Laboratory at December 31, 1984 and its current funds revenue and expenses and the changes in fund balances for the year then ended, in conformity with generally accepted accounting principles applied on a basis consistent with that of the preceding year.

Coopers & Lybrand

Boston, Massachusetts
April 29, 1985

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1984 and 1983

Assets	*1984*	*1983*
Cash and savings deposits	$ 214,449	$ 88,099
Money market securities (Note F)	825,000	550,000
Accounts receivable, net of allowance for uncollectible accounts	278,529	209,470
Receivables due for costs incurred on grants and contracts	395,788	500,872
Other assets	14,855	8,031
Total current assets	1,728,621	1,356,472
Investments, at cost (Note F)	8,020,168	7,340,392
Land, buildings and equipment (Note C)	17,436,604	17,196,770
Less accumulated depreciation	6,090,140	5,633,746
	11,346,464	11,563,024
Total assets	$21,095,253	$20,259,888

Liabilities and Fund Balances	*1984*	*1983*
Accounts payable and accrued expenses	$ 273,973	$ 360,758
Deferred income	95,519	62,857
Total current liabilities	369,492	423,615
Current unrestricted funds	13,952	149,161
Current restricted funds:		
Unexpended grants	410,210	86,114
Unexpended gifts	305,837	355,649
Unexpended income of endowment funds	18,736	43,178
	734,783	484,941
Endowment funds:		
Unrestricted purposes	1,771,188	1,716,309
Restricted purposes	1,432,604	750,434
	3,203,792	2,466,743
Quasi-endowment funds:		
Unrestricted purposes	1,020,338	865,619
Restricted purposes	2,692,957	2,752,141
	3,713,295	3,617,760
Plant funds:		
Unrestricted	11,346,464	11,489,019
Restricted	105,748	218,991
	11,452,212	11,708,010
Retirement fund balance (Note D)	1,607,727	1,409,658
Total liabilities and fund balances	$21,095,253	$20,259,888

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CURRENT FUNDS REVENUE AND EXPENSES

for the years ended December 31, 1984 and 1983

	Unrestricted		*Restricted*		*Total*	
	1984	*1983*	*1984*	*1983*	*1984*	*1983*
Revenue						
Grant reimbursement of direct costs:						
Instruction			$ 301,540	$ 413,797	$ 301,540	$ 413,797
Research			3,683,186	2,707,011	3,683,186	2,707,011
Recovery of indirect costs related to research and instruction:						
Summer program	$ 492,309	$ 428,535			492,309	428,535
Year-round program	1,572,260	1,342,117			1,572,260	1,342,117
Other	29,453	52,298			29,453	52,298
Instruction	35,131	33,331			35,131	33,331
Tuition	—	—	247,685	182,217	247,685	182,217
Support activities:						
Dormitory	497,263	434,686			497,263	434,686
Dining Hall	242,404	247,687			242,404	247,687
Library	227,551	184,325			227,551	184,325
Biological Bulletin	112,134	96,703			112,134	96,703
Research services	400,284	353,051			400,284	353,051
Marine resources	165,000	96,104			165,000	96,104
Other	472	(5,786)			472	(5,786)
Total support activities	1,645,108	1,406,770			1,645,108	1,406,770
Investment income	278,250	232,145	261,257	92,501	539,507	324,646
Availing of quasi-endowment fund balance			186,064	—	186,064	—
Gifts	92,693	257,067	451,135	664,190	543,828	921,257
Miscellaneous revenue	78,082	91,559			78,082	91,559
Total revenue	4,223,286	3,843,822	5,130,867	4,059,716	9,354,153	7,903,538
Expenses:						
Instruction			787,063	777,994	787,063	777,994
Research	71,038	55,274	4,220,462	3,196,866	4,291,500	3,252,140
Scholarships and stipends			190,280	195,714	190,280	195,714
Support activities:						
Dormitory	254,695	209,176			254,695	209,176
Dining Hall	245,141	235,149			245,141	235,149
Library	440,548	323,660	11,952	76,331	452,500	399,991
Biological Bulletin	158,127	120,822			158,127	120,822
Research services	635,897	543,422			635,897	543,422
Marine resources	325,660	271,224			325,660	271,224
Administration	979,045	959,831			979,045	959,831
Plant operation	976,605	903,729	11,387	3,516	987,992	907,245
Other	—	—	8,516	—	8,516	—
Total support activities	4,015,718	3,567,013	31,855	79,847	4,047,573	3,646,860
Total expenses	4,086,756	3,622,287	5,229,660	4,250,421	9,316,416	7,872,708
Unrestricted support of instruction	(98,793)	(174,727)	98,793	174,727	—	—
Excess (deficit) of revenue over expenses	$ 37,737	$ 46,808	—	$ (15,978)	$ 37,737	$ 30,830

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CHANGES IN FUND BALANCES

for the years ended December 31, 1984 and 1983

		Current Restricted Funds			*Endowment Funds*	
	Current Unrestricted Fund	*Unexpended Grants*	*Unexpended Gifts*	*Unexpended Income of Endowment Funds*	*Unrestricted*	*Restricted*
Balances at beginning of year	$ 149,161	$ 86,114	$355,649	$43,178	$1,716,309	$ 750,434
Increases:						
Unrestricted current funds revenue	3,852,343					
Grants for research		5,280,084				
Grants for instruction		329,829				
Gifts	92,693		283,312			544,732
Investment income	278,250		5,501	246,691		
Realized net gains on sale of investments			14,842		54,879	81,963
Addition to retirement fund						
Tuition		247,685				
Decreases:						
Instruction, research and support activities	(4,185,549)	(4,363,128)	(310,542)	(271,133)		
Indirect costs		(1,170,374)				
Payments to pensioners						
Depreciation						
Realized net gain (loss) on disposal of real estate investment						
Net change in fund balances before transfers	37,737	324,096	(6,887)	(24,442)	54,879	626,695
Transfers to (from) fund balances:						
Acquisition of fixed assets	(172,946)					
Other			(42,925)			55,475
Balances at end of year	$ 13,952	$ 410,210	$305,837	$18,736	$1,771,188	$1,432,604

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CHANGES IN FUND BALANCES

for the years ended December 31, 1984 and 1983

	Quasi-Endowment Funds		*Plant Funds*				
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*	*Retirement Funds*	*1984 Total All Funds*	*1983 Total All Funds*
Balances at beginning of year	$ 865,619	$2,752,141	$11,489,019	$218,991	$1,409,658	$19,836,273	$19,549,248
Increases:							
Unrestricted current funds revenue						3,852,343	3,412,282
Grants for research						5,280,084	4,015,187
Grants for instruction						329,829	396,280
Gifts				40,200		960,937	745,184
Investment income					88,223	618,665	505,836
Realized net gains on sale of investments	138,739	126,880			50,103	467,406	302,045
Addition to retirement fund					105,328	105,328	87,244
Tuition						247,685	182,217
Decreases:							
Instruction, research and support activities		(186,064)			(6,300)	(9,322,716)	(7,898,163)
Indirect costs						(1,170,374)	(974,640)
Payments to pensioners					(39,285)	(39,285)	(46,158)
Depreciation			(456,394)			(456,394)	(430,323)
Realized net gain (loss) on disposal of real estate investment	15,980					15,980	(9,966)
Net change in fund balances before transfers	154,719	(59,184)	(456,394)	40,200	198,069	889,488	287,025
Transfers to (from) fund balances:							
Acquisition of fixed assets			313,839	(140,893)		—	—
Other				(12,550)		—	—
Balances at end of year	$1,020,338	$2,692,957	$11,346,464	$105,748	$1,607,727	$20,725,761	$19,836,273

NOTES TO FINANCIAL STATEMENTS

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and nature history.

B. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with specified activities or objectives. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the donor or grantor of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating or other purposes. Unrestricted current funds are reported as revenue when earned.

Endowment funds are subject to restrictions requiring that the principal be invested with income available for use for restricted or unrestricted purposes by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, the principal of these funds may be expended for various restricted and unrestricted purposes.

Reclassifications

The financial statements for 1984 reflect certain changes in classification of revenue, expenses, and changes in fund balances. Similar reclassifications have been made to amounts previously reported in order to provide consistency of the financial statements.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of their proportionate share at market value adjusted for the cost of any additions or disposals.

C. *Land, Buildings and Equipment:*

Following is a summary of the unrestricted plant fund assets:

	1984	*1983*
Land	$ 689,660	$ 763,660
Buildings	14,772,449	14,480,320
Equipment	1,974,495	1,952,790
	17,436,604	17,196,770
Less accumulated depreciation	6,090,140	5,633,746
	$11,346,464	$11,563,024

Depreciation is computed using the straight-line method over estimated useful lives of fixed assets.

D. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. During 1984, the Laboratory changed its actuarial method used to determine pension cost from the aggregate cost method to the projected unit credit method. This change resulted in a decrease of $22,275 in the minimum contribution level. The Laboratory also revised certain actuarial assumptions to better reflect experience, which resulted in an additional decrease of $26,280 in the minimum contribution level.

The Laboratory's policy is to fund pension costs accrued, as determined under the projected unit credit method. The actuarially determined pension expense charged to operations in 1984 was $105,328. As of the latest valuation date, based on benefit information at December 31, 1983, the actuarial present values of vested and nonvested benefits, assuming an investment rate of return of 7%, were approximately $986,737 and $30,229, respectively. At December 31, 1984, net assets of the plan available for benefits were approximately $1,680,562.

In addition, the Laboratory has a pension plan funded by contributions to the Teachers Insurance and Annuity Association.

E. *Pledges and Grants:*

As of December 31, 1984 and 1983, the following amounts remain to be received on gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31, 1984		*December 31, 1983*	
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*
1984			$65,000	$293,285
1985	$ 5,000	$ 8,000	—	83,900
1986	5,000	8,000	—	3,965
1987	—	6,651	—	—
	$10,000	$22,651	$65,000	$381,150

In February 1979, the Laboratory initiated the MBL Second Century Fund, a phased effort, to secure $23,000,000 in support of capital rehabilitation, new construction, and endowment funds. As of December 31, 1984, the Laboratory has received pledges related to this effort of approximately $5,923,358, of which a substantial portion has been collected.

F. *Investments:*

The following is a summary of the cost and market value of investments at December 31, 1984 and 1983 and the related investment income and disposition of investment income for the years ended December 31, 1984 and 1983.

	Cost		*Market*		*Investment Income*	
	1984	*1983*	*1984*	*1983*	*1984*	*1983*
Endowment, Quasi-Endowment, and Pension						
U.S. Government securities	$2,422,701	$2,352,911	$2,405,964	$2,275,449	$272,442	$206,041
Corporate fixed income obligations	749,926	397,871	707,678	348,713	47,570	32,836
Common stocks	4,073,866	4,165,295	4,977,757	5,522,238	203,158	208,454
Preferred stock	12,549	—	22,940	—	1,798	
Money market securities	745,377	406,766	745,377	406,766	82,636	48,522
Real estate	15,749	17,549	15,749	17,549	—	—
Total	8,020,168	7,340,392	8,875,465	8,570,715	607,604	495,853
Less custodian fees					(32,331)	(30,282)
					575,273	465,571
Unrestricted Current Fund						
Money market securities	825,000	550,000	825,000	550,000	43,392	40,265
Total investments	$8,845,168	$7,890,392	$9,700,465	$9,120,715	$618,665	$505,836
Disposition of investment income:						
Restricted for current use:						
Utilized in current operations					261,257	92,501
Available for future operations					(9,065)	113,441
Total restricted current and quasi-endowment funds					252,192	205,942
Retirement fund					88,223	67,749
Unrestricted—utilized in current operations					278,250	232,145
					$618,665	$505,836

At December 31, 1984 and 1983 the following summarizes the participation of the various funds in the investment pool:

	1984	*1983*
Unexpended income of endowment	$ 18,736	$ 43,178
Unrestricted endowment	1,771,188	1,716,309
Restricted endowment	1,287,561	682,475
Unrestricted quasi-endowment	1,077,988	846,880
Restricted quasi-endowment	2,364,407	2,788,073
Retirement	1,500,288	1,263,477
	$8,020,168	$7,340,392

G. *Interfund Borrowings:*

Interfund balances at December 31 are as follows:

Current Funds	*1984*	*1983*
Due to retirement fund	$(105,328)	$(140,825)
Due to plant funds	(105,748)	(218,891)
Due to endowment funds	(500,000)	—
Due from (to) quasi-endowment funds	103,365	(43,520)
	$(607,711)	$(403,236)

VIII. REPORT OF THE LIBRARIAN

The 10th Annual Meeting of the International Association of Marine Science Libraries and Information Centers was held in Woods Hole in October. Over 120 participants attended the three-day sessions which featured 30 speakers, four workshops, a Cape Cod Clam Bake, and a Whale Watch. The subject of one of the discussions was the Use Survey conducted by the MBL through funding provided by the Rockefeller Foundation, and coordinated by Catherine Norton, Assistant Librarian.

The survey covered a ten-month period in 1983, March through the end of December. This was not a random sample which is a method used in most surveys; usage was studied by checking ALL journals used EVERY day for ten months. In January of 1984 staff members worked through all four stacks counting each volume that had been marked during the survey, and those numbers, combined with the numbers removed from the single issues that were used in the Reading Rooms, were fed into a computer. The total verified count showed that the collection was referred to 115,048 times.

The results in brief: 53% of the 4765 journal titles held by the Library were used during this time period. 76% of total usage involved the years 1980 through 1983. Use of periodicals by years of publication broke down as follows:

1980 through 1983	86,962
1961 through 1979	21,955
1941 through 1960	3,325
1901 through 1940	2,142
Pre 1900	624

Two studies were made to determine the depth of the collection; both studies used citation analysis. The first study examined the citations of several hundred articles published by both MBL and WHOI scientists and the second study involved citations from two Doctoral Dissertations. A PhD thesis on sedimentology by a WHOI student cited 89 journals covering the years 1914–1983. The Library owns 77 of them. The MBL thesis on invertebrate taxonomy cited 369 titles covering the years 1785–1982. The Library owns 336 of them.

More detailed reports will be published in the near future but for general interest we are listing the titles that were used 200 or more times during this period. It should be noted that this is what was used during ten months of a 96-year history. It would be most interesting to make the same study in 1994.

Journal Use Study at the MBL Journals Used 200 or More
Times During the 10-Month Period 04/83–01/84

NATURE	2938
SCIENCE	1709
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES	1681
SCIENCE CITATION INDEX	1517
BIOCHIMICA ET BIOPHYSICA ACTA	1267
BIOLOGICAL ABSTRACTS	1146
JOURNAL OF CELL BIOLOGY	1131
MARINE BIOLOGY	1091
JOURNAL OF BIOLOGICAL CHEMISTRY	1044
BIOLOGICAL BULLETIN, THE	1014
CURRENT CONTENTS; LIFE SCIENCES	1000
SCIENCE 83	975
JOURNAL OF PHYSIOLOGY	935
DEEP SEA RESEARCH	920

DEVELOPMENTAL BIOLOGY	812
CELL	793
JOURNAL OF GEOPHYSICAL RESEARCH	773
JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY	719
COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY	700
NEW SCIENTIST	696
BRAIN RESEARCH	692
NEW ENGLAND JOURNAL OF MEDICINE	654
MARINE ECOLOGY—PROGRESS SERIES	624
LIMNOLOGY AND OCEANOLOGY	612
EXPERIMENTAL CELL RESEARCH	569
BIOCHEMISTRY	564
BIOCHEMICAL PHARMACOLOGY	559
LANCET	559
JOURNAL OF COMPARATIVE PHYSIOLOGY	528
INDEX MEDICUS	498
JOURNAL OF MOLECULAR BIOLOGY	476
JOURNAL OF GENERAL PHYSIOLOGY	465
JOURNAL OF THE MARINE BIO ASSOC OF THE UK	458
PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON	454
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EARTH AND PLANETARY SCIENCE LETTERS	438
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BULLETIN OF MARINE SCIENCE	253
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IX. EDUCATIONAL PROGRAMS

SUMMER

Biology of Parasitism

Course director

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Other faculty, staff, and lecturers

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Cross, George, Rockefeller University
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Neural Systems and Behavior

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Physiology

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SPRING

Biophysics of Neural Function

Course director

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SHORT COURSES

ANALYTICAL AND QUANTITATIVE LIGHT MICROSCOPY

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BASIC IMMUNOCYTOCHEMICAL TECHNIQUES IN TISSUE SECTIONS AND WHOLE MOUNTS

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CELLULAR NEUROBIOLOGY IN THE LEECH

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MARICULTURE: CULTURE OF MARINE INVERTEBRATES FOR RESEARCH PURPOSES

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OPTICAL MICROSCOPY AND IMAGING IN THE BIOMEDICAL SCIENCES

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PROTEIN ANALYSIS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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Small Computers in Biology and Medicine

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Woodwell, George M. (on sabbatical as of 2/1/84)

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Hobbie, John E. (as of 2/1/84)

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Glick, Stephen
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Griffin, Elisabeth
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Johnson, Stephen
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Montgomery, Mary Louise
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Rossi, Mary Ann
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Stone, Thomas
Turner, Andrea R.
White, David

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Nadelhoffer, Knute, University of Wisconsin
Rudnick, David, University of Rhode Island

Visiting investigator

Luther, George, Kean College of New Jersey

XI. HONORS

Friday Evening Lectures

Nelson, George S., Liverpool School of Tropical Medicine, 29 June, *"Wild Animals as Hosts of Human Disease in Africa"*

Gainer, Harold, National Institutes of Health, 6 July, Lang Lecture, *"The Brain as a Secretory Organ: the Endocrine Neuron"*

Colwell, Rita R., University of Maryland, 13 July, Jerome K. Merlis Memorial Lecture, *"Marine Biotechnology—Marine Biology Loses Its Innocence and Enters the Maelstrom of Genetic Engineering"*

Hille, Bertil, University of Washington, 19, 20 July, Forbes Lectures, I. *"Ionic Channels of*

Excitable Cells: Fitness, Diversity and Evolution;" II. *"Ionic Channels are Gated Macromolecular Pores"*

McLaren, Anne, MRC Mammalian Development Unit, University College, London, 27 July, *"The Human Embryo: Landmarks in a Continuum"*

Ehinger, Berndt, University of Lund, 3 August, *"Retinal Circuitry from an Ophthalmologist's Viewpoint"*

Altman, Sidney, Yale University, 10 August, *"A Novel Catalytic Role for RNA"*

Kennedy, Donald, Stanford University, 17 August, *"Let's You and Him Fight: Government Policies and the Cost of Doing Research"*

Stephens, Ray, Marine Biological Laboratory, 24 August, *"The Tubulins: Building Blocks for Microtubules of Varied Function"*

May, Robert, Princeton University, 31 August, *"How to Host a Parasite and Other Ecological Lessons"*

ASSOCIATES' LECTURE

Robinson, Denis, 18 August, *"The Preparation of Young Minds"*

SPECIAL LECTURE

Wald, George, Harvard University, 11 July, *"Life and Mind in the Universe"*

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TORO, LIGIA G. CALDAZA, Universidad Autonoma Metropolitana—Iztapalapa, Mexico

XII. INSTITUTIONS REPRESENTED

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Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional, Mexico
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Institute for Medical Research, Malaysia
Institute of Oceanology, Peoples' Republic of China
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Liverpool School of Tropical Medicine, England, U. K.
Lund, University of, Sweden
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McGill University, Canada
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Memorial University of Newfoundland, Canada
Montreal Neurological Institute, Canada
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Tjarno Marine Biological Laboratory, Sweden
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Tokyo, University of, Japan
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Zoologishes Institute, Zurich, Switzerland

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THE EFFECTS OF TEMPERATURE AND ACCLIMATION ON CRUSTACEAN NERVE-MUSCLE PHYSIOLOGY

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ABSTRACT

Crustaceans, like most ectotherms, have body temperatures that are close to ambient. Although some animals live in constant warm conditions, most crustaceans live in environments with both short- and long-term temperature fluctuations. Rapid temperature changes generally produce changes in the properties of the nerve and muscle membranes. As a result increases in temperature generally cause a decrease in the effectiveness of neuromuscular transmission. This is offset by an increase in the amount of synaptic facilitation, an increase in axon firing frequencies, and in some motor axons the production of additional responses in the peripheral branches. Although these changes act to overcome temperature-induced decreases in muscle tension, little is known about how the intact animal utilizes these changes to produce coordinated movements at different temperatures.

Long-term changes in thermal conditions alter the properties of the motor nerves and the muscles. This results in a shift in the temperature range over which there is optimal neuromuscular performance towards the acclimation temperature.

INTRODUCTION

Many endotherms are well-insulated and are able to retain much of the heat produced during metabolism, so that body temperature is maintained within carefully defined limits. Ectotherms, by contrast, have a high thermal conductance so that any heat produced is quickly lost to the environment. As a result the internal temperature of many ectotherms is close to the ambient temperature. The problem faced by most ectotherms, therefore, is one of retaining a degree of functional integrity in a thermally fluctuating environment.

Certain ectotherms overcome this problem by living in an environment that provides near-constant thermal conditions. Hawaiian ghost crabs, for example, live in burrows and in the ocean at temperatures between 26 and 28°C, and their nocturnal habits ensure that they do not overheat in the sun. These crabs, therefore, utilize only minor behavioral modifications to maintain a near-constant body temperature. In the laboratory, however, exposure to cool temperatures results in these animals becoming sluggish and ultimately immobile with muscles that exhibit fibrillation, while warm temperatures cause jerky and erratic limb movements (Florey and Hoyle, 1976).

At the other extreme, certain ectotherms live in environments with temperatures that fluctuate dramatically. Animals that live in such environments must not only be able to withstand both short-term (daily) and long-term (seasonal) temperature changes, but must also remain functional over a wide thermal range. For example, the neuromuscular systems of shore crabs (*Pachygrapsus*) and crayfish (*Astacus*) can function over 15 and 24°C ranges, respectively (Harri and Florey, 1977; Stephens and Atwood,

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1982). It is apparent that in these animals certain compensatory mechanisms must be present that permit the animal to function over a wide temperature range. In crayfish, Kivivouri (1980) has shown that different neuronal pathways exhibit different resistances to short-term temperature changes. In general, complex reflexes are more sensitive to temperature change than simple reflexes, synaptic function is more sensitive than nerve conduction, and inhibitory junctions are more sensitive than excitatory (White, 1983). These results are similar to those described in goldfish (Prosser and Fahri, 1965; Prosser and Nagia, 1968; Friedlander *et al.*, 1976). In addition to withstanding rapid changes in temperature, ectotherms must also tolerate long-term thermal stress. In crayfish, for example, acclimation causes a shift in the lethal temperature (White, 1983) and modifies the temperature at which optimal activity takes place (Kivivouri, 1983). Moreover, when placed in an experimental regime in which animals can select their own environmental temperature, it has been shown that active animals prefer their ambient temperatures (Taylor, 1984, but see Crawshaw, 1983). Therefore, it is apparent certain mechanisms do exist that enable animals to modify their behavioral output in response to long-term changes in temperature.

The aim of the present paper is to describe the effects of short- and long-term temperature changes on the physiology of nerves and muscles, with special reference to crustaceans that normally live in a thermally fluctuating environment. Most of the work published to date has used neuromuscular preparations in walking limbs or claws. Since little work has been published that describes recordings from intact animals, most of the results outlined in this article were obtained using autotomized limb preparations which were bathed in a physiological saline, and whose temperature was carefully controlled and monitored.

Muscle Recordings

Resting potential

In crustacean muscle fibers the membrane potential increases with temperature. In crayfish, the membrane potential changes exhibit two linear components (Harri and Florey, 1977, 1979), whereas in crabs there are two logarithmic-linear components (Stephens and Atwood, 1982; Stephens, 1985). In both animals, the component observed at cooler temperatures has a steeper slope, and in some cases the change in membrane potential takes place faster than would be predicted by the Nernst equation. It has been suggested that the two components may be explained in terms of differential temperature effects on the membrane conductance to potassium and chloride, relative to sodium (Harri and Florey, 1977, 1979). Membrane potential recordings from crab and crayfish muscle fibers have revealed that the temperature at which the two components intersect can be changed by altering the acclimation temperature (Harri and Florey, 1979; Stephens and Atwood, 1982). Acclimation to different temperatures causes changes in the fluidity and saturation of lipids in membrane of goldfish (Roots and Johnston, 1968; Driedzic *et al.*, 1977; Matheson *et al.*, 1980), and crustaceans (Chapelle, 1977, 1978; Chapelle *et al.*, 1979). Thus, acclimation could produce certain fundamental changes in membrane composition, which may differentially influence the operation of channels for potassium, sodium, and chloride ions, as well as those concerned with leakage. Such a differential effect on the relative permeabilities of a membrane to different ions has been demonstrated in barnacles (Dipolo and Latorre, 1972; Fischbarg, 1972). Recently, it has been shown that the membrane potential can hyperpolarize beyond the potassium potential at high temperatures (White, 1983). This has been explained in terms of temperature-induced changes in the properties of the sodium-potassium pump, producing a change in the distribution of these ions across the membrane (Florey and Hoyle, 1976). Evidence for this comes from the observation that the rate of spontaneous motor neuron firing can be changed by temperature-induced changes in the sodium pump (Arechiga and Cerbon, 1981).

Irrespective of the mechanism that produces the two-phase relationship between temperature and membrane potential, one interesting phenomenon is that cold temperatures cause the muscle membrane to become depolarized above the excitation-contraction threshold, which is independent of temperature (Dudel and Ruedel, 1968), and creates passive tension in the muscle (Harri and Florey, 1977; Fischer and Florey, 1981). The amount of tension produced is dependent upon the amount of depolarization (Orkand, 1962) and can be released by direct action of the inhibitor on the muscle fiber. This suggests that post-synaptic inhibition may play a significant role in the behavior of an animal at cold temperatures.

Excitatory junctional potentials

In many crustacean muscle fibers the amplitude and the time course of the nerve evoked excitatory junction potential (ejp) (Fig. 1A, B) and the tension produced in the muscle decline with temperature (Harri and Florey, 1977; Stephens and Atwood,

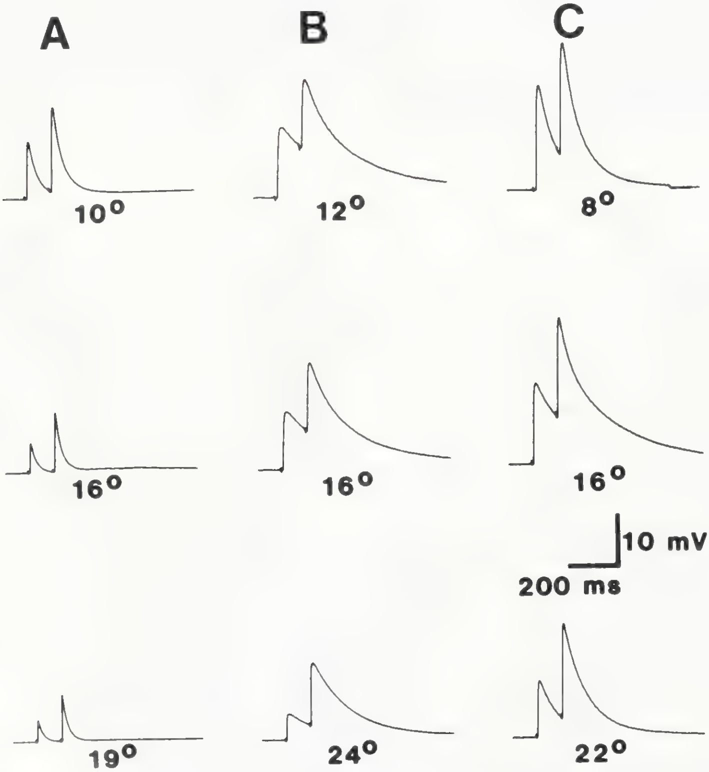

FIGURE 1. Examples of the effect of temperature on ejp's recorded from three different types of fibers in the stretcher muscle in a walking leg of *Pachygrapsus crassipes*. The pen recordings show changes in amplitude, time course, and facilitation, with a 100 ms interpulse interval (from Stephens and Atwood, 1983).

1983; White, 1983). The change in ejp time course can be fully accounted for by changes in the membrane conductance (Fischer and Florey, 1981). The decline in muscle tension with temperature is considered to be due to three factors: (1) depolarization of the membrane potential away from the temperature independent excitation-contraction threshold; (2) decline in ejp amplitude resulting in a smaller depolarization of the membrane following each impulse; and (3) decline in ejp time course which results in less summation of successive ejp's. These three factors result in a decline in neuromuscular efficacy with temperature.

In some muscle fibers ejp amplitude does not simply decline with temperature, but shows an initial increase followed by a decline (Fig. 1C). Examples of axons with this feature include the slow closer excitor to the crayfish closer muscle (Harri and Florey, 1977), the frog sartorius neuromuscular junction (Jensen, 1976; White, 1976), the rat phrenic-diaphragm (Hubbard *et al.*, 1971; Ward *et al.*, 1972), and the stretcher excitor and the slow bender excitor in walking legs of the Californian shore crab (*Pachygrapsus crassipes*) (Stephens and Atwood, 1982; Stephens, 1985). In certain stretcher muscle fibers the ejp amplitude and time constant have maximum values at the acclimation temperature (Stephens and Atwood, 1982). It has been suggested that the decline in ejp amplitude at cold temperatures may be due to conduction block in certain nerve branches (Hatt and Smith, 1975; Lang and Govind, 1977), or to temperature-induced changes in quantal content (White, 1983) perhaps produced by altering the amount of calcium entering the terminal (Charlton and Atwood, 1979).

In the crab stretcher muscle the degree of ejp facilitation, measured by comparing the relative amplitudes of pairs of responses evoked at different time intervals, is dependent upon temperature. Around the acclimation temperature, when the amplitude and time course of the ejp are at a maximum, facilitation (at frequencies > 10 Hz) is at a minimum (Stephens and Atwood, 1982). Changes in temperature cause a decline in ejp amplitude and time constant, but an increase in facilitation. As a result, the amount of tension produced by the muscle in response to short trains of excitatory axon spikes remains approximately constant within an 8°C range around the acclimation temperature. In this way the neuromuscular apparatus of the crab compensates for small temperature fluctuations.

It is interesting that the slow excitor to the bender muscle produces ejp's with some features that are different from those recorded from the antagonistic stretcher muscle. Bender muscle ejp's have maximum amplitudes and exhibit minimum facilitation around the acclimation temperature, as in the stretcher. However, increases in temperature result in a decline in the ejp time course, which can be fully accounted for by changes in the membrane input resistance (Stephens, 1985); an inverse relationship between membrane resistance and temperature has been reported in other crustacean muscles (Fatt and Katz, 1953; Fischer and Florey, 1981; White, 1983). Interestingly, the amount of tension produced by the bender muscle does not exhibit a temperature range over which it remains constant but also simply declines with temperature. These data suggest that tension is more closely linked to ejp time course than to amplitude. How the differential production of tension in antagonistic muscles affects the behavior of the crab at different temperatures has not been defined.

The stretcher muscle is innervated by a single excitatory motor neuron (Wiersma and Ripley, 1952), which has a diverse array of synaptic types (Fig. 1; Atwood, 1967). It is possible, therefore, to record from some fibers with synapses that produced small ejp's and good facilitation, and from other fibers with large amplitude ejp's that exhibit defacilitation or depression (Atwood and Bittner, 1971). The amount of facilitation or depression can be measured by producing pairs of ejp's at different intervals and

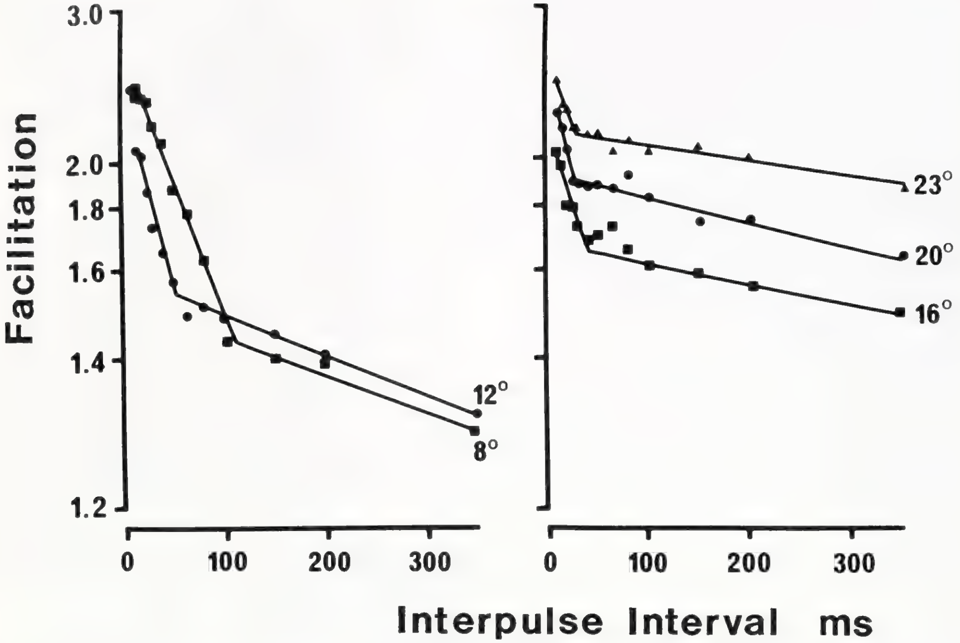

FIGURE 2. The effect of temperature on a highly facilitating muscle fiber in the stretcher muscle in a walking leg of *Pachygrapsus crassipes*. Facilitation was measured by producing pairs of ejp's at different intervals and comparing the amplitude of the second with the first response (from Stephens and Atwood, 1983).

comparing the amplitude of the second response with the first. When the measurements are corrected for non-linear summation (Martin, 1955), the values are very similar to those obtained from extracellular recordings of synaptic currents from the same fiber (Stephens and Atwood, 1983). In fibers with small amplitude ejp's, the amount of facilitation decays as the time interval between the ejp's is increased (Fig. 2). This decay in the amount of facilitation can be divided into two logarithmic-linear components (F_1 and F_2), which have been explained in terms of a decline in the probability of transmitter release following stimulation, possibly related to two different processes for the removal of calcium (Kita *et al.*, 1980). Increasing temperature causes the slope of F_1 to increase, the slope of F_2 to first increase and then decrease, and the time interval at which the two components intersected to decrease (Fig. 2). Thus, in these types of fibers, as temperature is increased the F_2 component becomes more dominant and the amount of facilitation shows an overall increase.

In the case of the large amplitude ejp's, temperature changes cause the facilitation profiles to change dramatically (Fig. 3). When ejp's are evoked at short time intervals, the amount of defacilitation decreases with increased temperature. When ejp's are elicited at longer time intervals, temperature increases result in defacilitation being replaced by facilitation. Extracellular recordings of focal synaptic currents from the muscle surface have revealed that this effect is not produced by temperature-induced changes in the efficacy of a heterogeneous population of synapses on single muscle fibers. Instead, synapses that produce large amplitude, de-facilitating ejp's at low temperatures change with increased temperature to produce small amplitude, facilitating

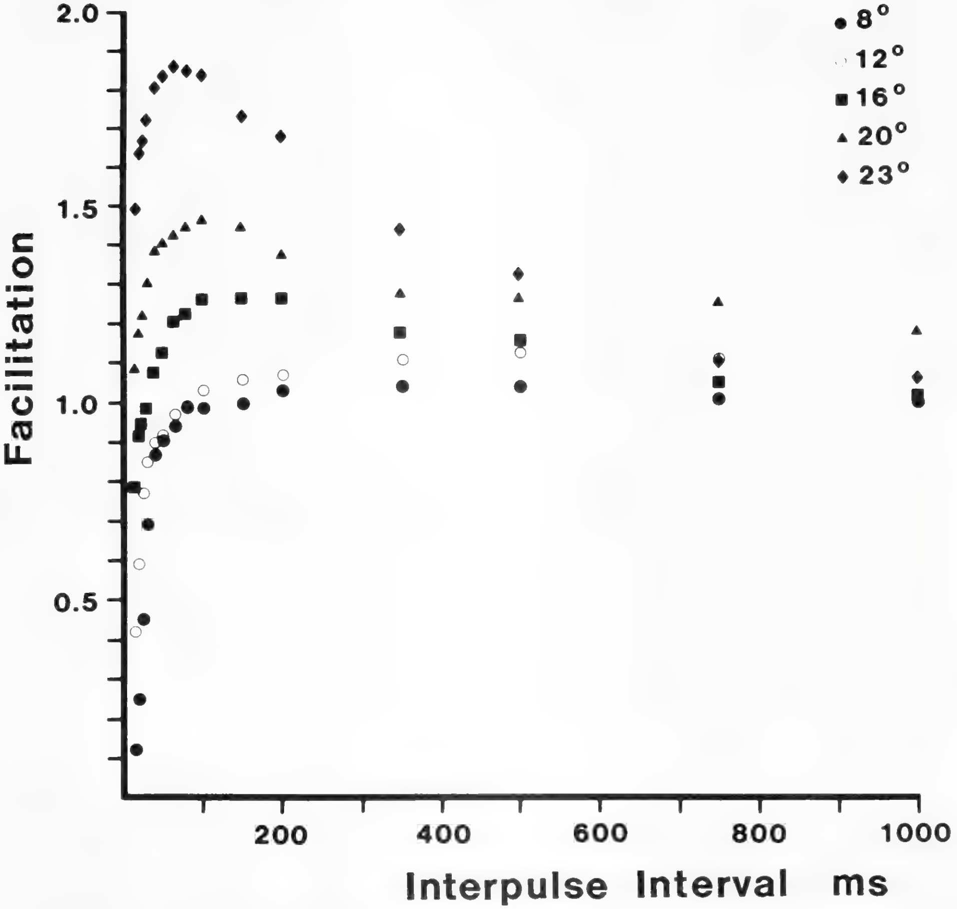

FIGURE 3. The effect of temperature on synaptic depression recorded from a single fiber in the stretcher muscle in a walking leg of *Pachygrapsus crassipes*. The graph shows the amount of facilitation at different temperatures. Facilitation was measured by producing pairs of ejp's at different intervals and comparing the amplitude of the second with the first response. Note that values > 1 indicate facilitation, while values < 1 indicate depression (from Stephens and Atwood, 1983).

ejp's. One possible mechanism to explain this observation is simply that increased temperature reduces spike duration. This has been shown in the axon in the merus (Stephens *et al.*, 1983) and in the terminals (Dixon and Atwood, pers. comm.). This temperature-induced decrease in spike duration would not only result in the release of less transmitter at the terminal and produce a small ejp, but would also leave more of the transmitter store available for release by the second action potential. However, broadening the spike with 3-aminopyridine (a potassium channel blocker) has little effect on the amount of facilitation observed at high temperature (Stephens and Atwood, 1983).

It is interesting that long-term facilitation, in which the amount of transmitter released at the synaptic terminals increases during maintained nerve activity, is also temperature sensitive (Jacobs and Atwood, 1981a). This type of facilitation occurs at its optimum at or below the acclimation temperature, suggesting that long-term facilitation may be one mechanism in which neuromuscular performance may be enhanced at low temperatures.

AXON RECORDINGS

Resting potential

As in muscle fibers, the axonal membrane potential declines with temperature (Dalton and Hendrix, 1962; Stephens, 1985). In the slow excitatory axon (E2) to the crab bender muscle there are two logarithmic-linear components, the component with the steeper slope being observed at cooler temperatures. As found in the muscle, the two components intersect at a temperature around acclimation (Stephens, 1985). The similar relationships between membrane potential and temperature for the motor axon and the muscle fibers indicate similar thermal effects on both membranes.

Action potential

In the crab motor axon (E2) to the bender muscle the amplitude and the time course of the action potential decline exponentially with temperature with Q_{10} values of 1.2 and 1.97, respectively (Stephens, 1985). The input resistance also declines with temperature (see also Colton and Freeman, 1975) with a Q_{10} of 2.0. This value is not significantly different from that obtained for the time course of the spike.

The axonal spike is followed by a hyperpolarizing after-potential in the lobster giant axon (Dalton and Hendrix, 1962) and a depolarizing after-potential in the E2 axon to the crab bender muscle (Stephens *et al.*, 1983). In both of these examples, the magnitude of the after-potential increases with temperature.

Peripheral generation of action potentials

In the Californian shore crab (*P. crassipes*) increasing the temperature above a critical threshold causes a single action potential in the stretcher excitor to produce more than one action potential and ejp (Fig. 4A; Stephens and Atwood, 1981). This effect can be reversed by cooling the preparation below the threshold temperature. The temperature threshold for this effect can be altered by acclimation (Stephens and Atwood, 1981) and by bath applications of low levels of ethanol (Stephens and Lazarus, 1981; Lazarus *et al.*, 1982). During a burst, the number of additional nerve spikes increases with temperature and is always matched with a concomitant number of ejp's (Fig. 4B–D—panel E). Moreover, the additional responses are produced at such a frequency that the ejp's undergo summation and facilitation and result in an increase in muscle tension (Stephens, 1985).

Extracellular recordings from two locations along the axon reveal that the additional spikes are generated in the periphery and travel antidromically down the axon (Stephens and Atwood, 1981). Similar observations have been made for the specific inhibitor to the stretcher muscle (Fig. 4B–D—panel SI) and the slow excitor (E2) to the bender muscle, the stretcher excitor of the green crab *Carcinus maenas* (Stephens, unpub. obs.), and in motor neurons in *Sh Drosophila* mutants (Ganetzky and Wu, 1982). Furthermore, observations that are consistent with this phenomenon have been recorded extracellularly from the bender muscle in intact shore crabs (Lazarus *et al.*, 1982).

The additional spikes that are generated in the periphery of the stretcher excitor axon can be modulated by activity in the specific inhibitor (Fig. 4). In this case, there is always a matching between the number of excitatory axon spikes and the number of ejp's (Fig. 4B–D—panel E + SI), suggesting that the point of spike modulation is

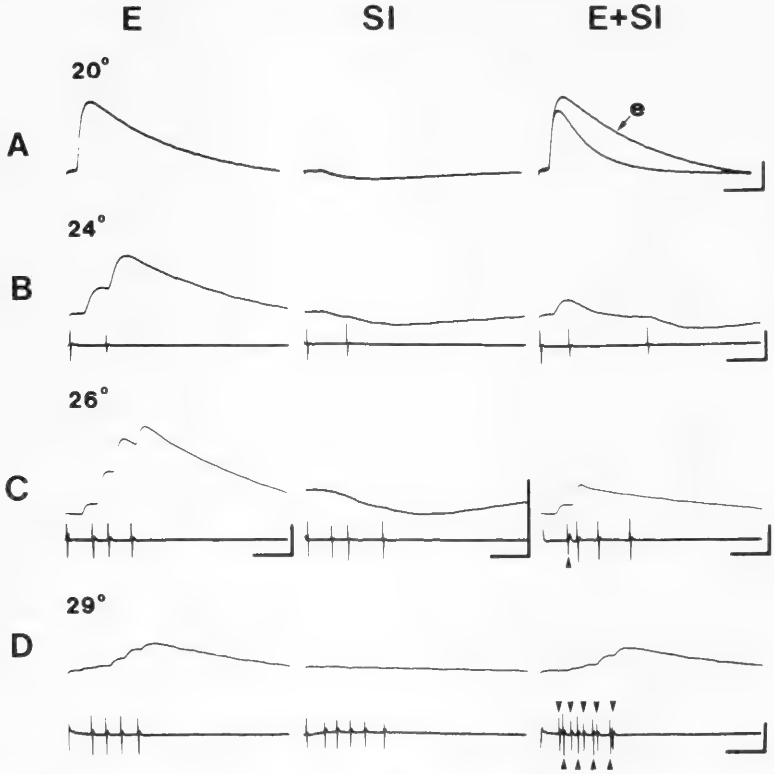

FIGURE 4. Junctional potentials and nerve spikes recorded following stimulation of the excitor (E) and the specific inhibitor (SI) axons to the stretcher muscle in an autotomized walking leg of *Pachygrapsus crassipes*. A. Recordings at 20°C showing the ejp (e) superimposed upon the response when both axons were stimulated simultaneously. B. Stimulation of either axon provoked junctional potentials and two nerve spikes. Synchronous stimulation of both axons abolished the second ejp and the second E axon spike. C. Stimulation of either axon provoked four junctional potentials and four axon spikes. Stimulation of SI after E abolished 2 ejp's and 2 E axon spikes. Note the E and SI spikes can be differentiated on the basis of contrasting amplitudes; the arrow points to the single antidromic E axon spike. D. At 29°C the SI spike-ijp coupling had broken down. Synchronous stimulation of both axons did not abolish the ejp's, but resulted in four peripherally generated E spikes (arrows below trace) and five peripherally generated SI spikes (arrows above trace). Calibration 5 mV and 10 ms (from Stephens and Atwood, 1981).

very close to the point where the additional spikes are generated. Moreover, the additional spikes can be abolished by bathing the preparation in gamma-aminobutyric acid, the inhibitory synaptic transmitter (Otsuka *et al.*, 1966), and the modulating effect of the inhibitor can be abolished by bath application of picrotoxin, an antagonist to inhibitory transmission (Takeuchi and Takeuchi, 1969). These data suggest that the points of spike generation and modulation are in the area of the axo-axonal synapses between the specific inhibitor and the excitor, possibly at excitatory axon branch points or "bottlenecks" (Jahromi and Atwood, 1974).

The phenomenon of peripheral spike generation has been recorded from the single excitor and the specific inhibitor to the crab stretcher muscle (Fig. 4) and from the slow (E2) excitor to the antagonistic bender muscle (Figs. 5E and 6C, D). It is interesting that action potentials recorded from the three axons consist of a spike followed by a depolarizing after-potential (Fig. 5B). Other axons that have a hyperpolarizing after-

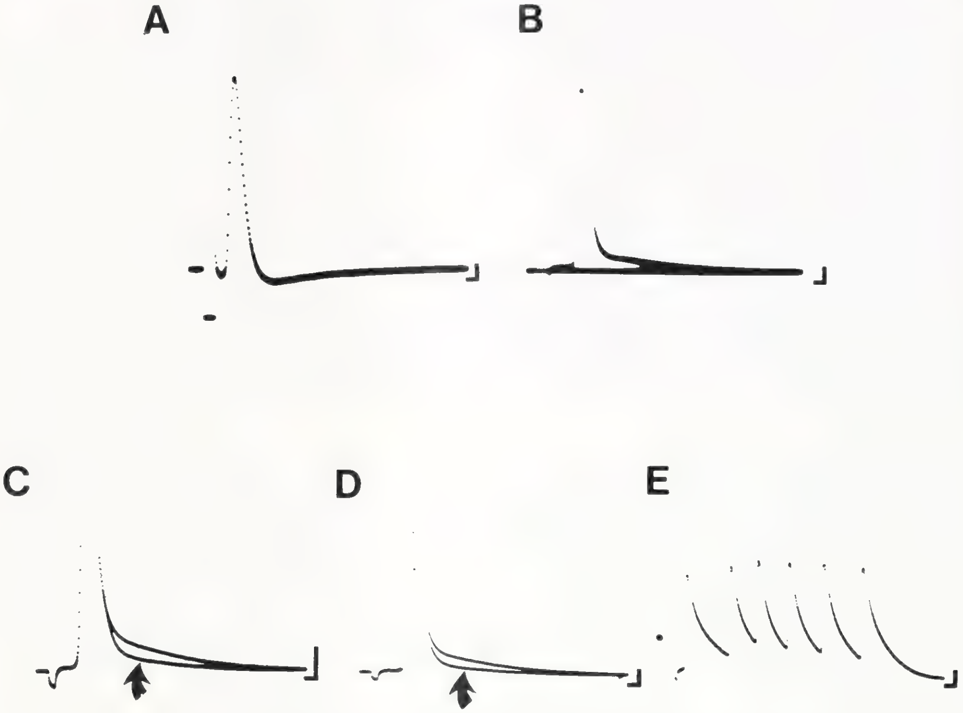

FIGURE 5. The effect of temperature and ethanol on the action potential recorded from excitatory axons to the bender muscle in an autotomized walking leg of *Pachygrapsus crassipes*. The action potentials recorded from E1 (A) and E2 (B) axons show hyperpolarizing and depolarizing after-potentials, respectively. Bathing preparations in saline containing 2% ethanol (C) or heating to a warm (26°C) temperatures (D) provoked an increase in the amplitude of the depolarizing after-potential in the E2 axon; traces are superimposed on an action potential recorded in normal saline at 14°C (arrow). Warming to 30°C resulted in a single axon shock provoking a train of E2 action potentials. Calibration 10 mV and 1 ms (from Stephens *et al.*, 1983).

potential, for example the fast excitatory (E1) axon to the bender muscle (Fig. 5A), do not exhibit peripheral spike generation. Increasing the temperature or bath applications of ethanol, both of which lower the temperature threshold for peripheral spike generation (Stephens and Lazarus, 1981), increases the size of the depolarizing after-potential (Fig. 5C, D). Further, elevating the temperature decreases the time course of the refractory period, so that at warm temperatures the elevated membrane potential produced by the depolarizing after-potential creates a period of decreased threshold (Stephens *et al.*, 1983). It has been suggested that the increased membrane resistance encountered where the axon becomes narrow, at branch points and "bottlenecks," increases the size of the depolarizing after-potential (Stephens *et al.*, 1983). If this is the case, immediately following the refractory period the increased membrane depolarization will be above threshold and additional action potentials will be generated at the periphery. Whether temperature influences the threshold for action potential production has not been established in crab axons.

If this hypothesis is correct, it is apparent that the generation of additional action potentials in the peripheral branches of axons is closely linked to the temperature-sensitive depolarizing after-potential. In crayfish and *Aplysia* neurons, the depolarizing after-potential has been explained in terms of an increase in calcium conductance (Yamagishi and Grundfest, 1971; Lewis, 1984). This is particularly interesting since calcium and sodium are also considered to be involved in the spike portion of the action potential (Yamagishi and Grundfest, 1971; Kawai and Niwa, 1980). Therefore, it seems that increased temperature affects the sodium and calcium currents to decrease the size of the spike, but has an influence on other calcium currents to increase the magnitude of the depolarizing after-potential (Stephens *et al.*, 1983). The mechanism underlying this apparent contradiction remains to be understood.

The generation of additional spikes in the peripheral branches of certain axons is one way in which neural firing patterns can be altered by temperature. Since the pattern of motor impulses influences the amount of tension produced by the muscle (Wiersma and Adams, 1949), this dramatic change in axon firing at high temperatures may compensate for the decreasing effectiveness of the neuromuscular junction and may be one mechanism whereby ectotherms extend their thermal range. However, if animals lack the ability to coordinate this additional activity the result will be a series of erratic and jerky movements, as described in Hawaiian ghost crabs at high temperatures (Florey and Hoyle, 1976). The temperature threshold for peripheral spike generation is not the same for the stretcher excitor and the E2 bender excitor (Fig. 6C). The number of ejp's and the frequency at which they are produced at a particular temperature are not always the same (Fig. 6D). Also, the additional spikes in the stretcher excitor can be modulated (Fig. 4B–D—panel E + SI) while those in the E2 axon can not (Lazarus *et al.*, 1982). As a result of these factors, it seems likely that this differential effect of temperature on the motor supplies to antagonistic muscles will create significant coordination problems in the intact animal. Additional problems will be encountered when two axons are involved, one that shows peripheral spike generation and the other that does not, as in the excitatory motor supply to the bender muscle (Stephens *et al.*, 1983). The extent of these coordination problems in the intact animal has not been established.

Axon firing patterns can be altered by temperature. This has been shown in sensory neurons (Hatt, 1983), interneurons (*e.g.*, Langley, 1979; Prior and Grega, 1982; Nelson and Prosser, 1981), and motor neurons (Arechiga and Cerbon, 1981; and see Fischer and Florey, 1981). Temperature-induced changes in the conduction velocity and firing patterns of neurons may alter the phases between oscillator neurons and feedback relationships and thereby change particular behavior patterns. This has been reported in the Hawaiian ghost crab, where running speed is closely correlated with temperature (Florey and Hoyle, 1976). It is apparent that certain ectotherms can compensate for temperature-induced changes in the effectiveness of nerve and muscle function with the result that they remain functional throughout a wide temperature range, however the mechanisms by which this is achieved are poorly understood.

CONSIDERATIONS FOR THE FUTURE

Based on the above data, one outstanding problem involves attempting to explain the mechanism underlying the two-phase relationship between temperature and membrane potential. It has been suggested that temperature may influence the sodium-potassium pump and/or have a differential effect on ion channels in the membrane. Answers to this problem could be obtained using techniques such as ion-selective electrodes, to examine whether the levels of potassium or chloride ions inside nerve and muscles are altered by changes in temperature. The use of voltage clamp techniques

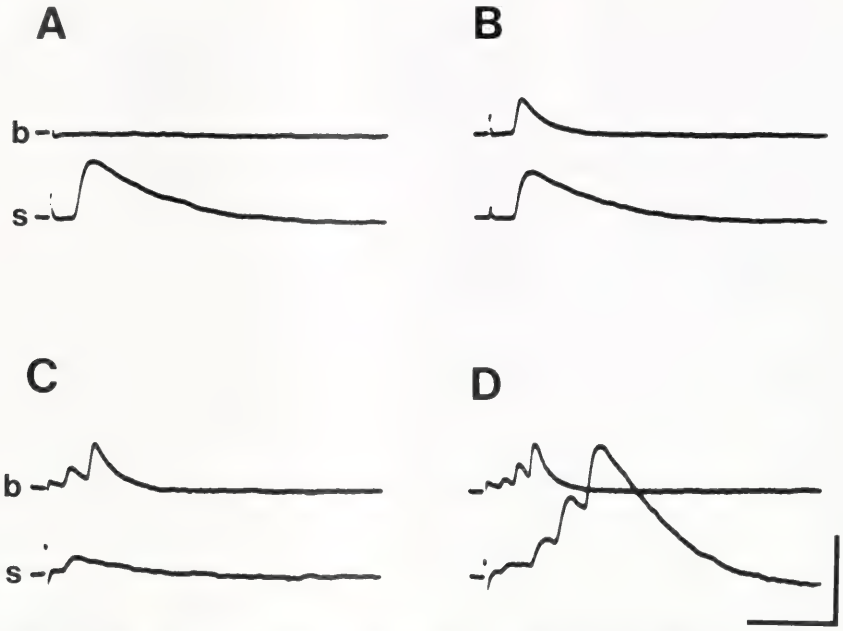

FIGURE 6. The different temperature thresholds for peripheral spike generation in excitor axons to the stretcher (s) and bender (b) muscles in an autotomized walking leg of *Pachygrapsus crassipes.* A. Low intensity stimulus shocks applied to the limb nerve evoked activity in the excitor to the stretcher and an ejp. B. Increasing stimulus intensity recruited the E2 axon to the bender muscle and evoked an ejp. C. At 28.5°C two ejp's were recorded from the bender muscle. D. At 30°C, three ejp's were recorded from the bender muscle and four ejp's from the stretcher muscle. Calibration 10 mV and 10 ms (from Lazurus *et al.*, 1982).

on axons is confined by size constraints, although this technique has been used on unidentified crustacean walking leg axons (Connor, 1975). In such a study, the ionic currents that are involved in the axon spike as well as the depolarizing after-potential could be defined.

One area that has received little attention to date involves the effects of temperature on inhibitory nerves. It is known that temperature increases result in inhibitory synapses failing earlier than excitatory (Fig. 4D; Stephens and Atwood, 1981; White, 1983). However, little is known about the effect of temperature on post- *versus* pre-synaptic inhibition, and the relative temperature-sensitivity of different inhibitors to the same muscle, for example the common inhibitor and specific inhibitors to certain crustacean limb muscles.

Studies on the effect of temperature on the firing patterns of neurons has generally been confined to sensory and motor neurons. Is it possible that temperature also influences neurons that secrete hormones, for example those that have a modulatory effect on neuromuscular transmission (Hoyle *et al.*, 1974; Jacobs and Atwood, 1981b)? This may have a profound effect on the behavior of the intact animal, more than would be predicted by examination of nerve-muscle preparations in autotomized limbs.

Finally, the effects of temperature on interneurons has been given only minimal attention, probably due to difficulties encountered when trying to routinely record from these neurons. Are the effects similar to those observed in the periphery, and, perhaps equally important, what are the effects of temperature on the properties of electrical synapses? These and many other questions must be answered if the effects of temperature on the behavior of cold-blooded animals are to be fully understood.

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CHEMICAL ATTRACTION CAUSING AGGREGATION IN THE SPINY LOBSTER, *PANULIRUS INTERRUPTUS* (RANDALL), AND ITS PROBABLE ECOLOGICAL SIGNIFICANCE

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ABSTRACT

Field and laboratory experiments were performed to determine the mechanisms of aggregation by the lobster, *Panulirus interruptus*. Capture-frequencies by unbaited traps, modified to simulate refuges by allowing lobsters to both enter and exit, were found to fit a negative binomial ($k < 0.5$) rather than a Poisson distribution. Such over-dispersion in the capture of lobsters was not fully attributable to environmental factors, suggesting that conspecific attraction may have been occurring. Laboratory trials conducted in a large rectangular tank (9.0 m × 2.4 m × 1.0 m) demonstrated that substances released by both sexes are highly attractive to conspecific males and females alike, resulting in aggregation. Abalone muscle, a potent feeding attractant to *P. interruptus,* was ineffective in initiating aggregation while dead lobsters, excised lobster thorax and abdominal muscle were all avoided. The tendency to aggregate changes during the course of a night, and aggregations are probably formed just before dawn and maintained until dusk. Results are consistent with a hypothesis that conspecific interactions facilitate anti-predatory defense and avoidance in *Panulirus.*

INTRODUCTION

Benefits of living in groups include a reduction in predation by concealment among cohorts (Williams, 1964; Hamilton, 1971), collectively increased prey vigilance (Pulliam, 1973; Treisman, 1975a, b), cooperative foraging (Schoener, 1971), increased reproductive success (Alexander, 1974), and competition leading to increased foraging efficiency (Pulliam, 1976). However, aggregation does not always imply benefit, or that gregarious behavior has occurred. Habitat patchiness and lack of refuges can also cause contagious spatial distributions.

The discovery of aggregation-causing attractions between individuals would give evidence for gregariousness. Although chemical signals are known to mediate social behavior in both terrestrial and aquatic arthropods, most previous investigations have emphasized those which lead to courtship and mating, that is sex pheromones (see Jacobson, 1972, and Dunham, 1978, for reviews). Other studies have reported colonization effects, as in beetles (*e.g.,* Wood, 1962; Birch, 1978; Borden, 1982, 1984) and in barnacles (*e.g.,* Crisp and Meadows, 1962; Rittschof *et al.,* 1984), in which chemical attraction has led to high conspecific densities in habitats that enhance survival. Chemical cues have also been found to specifically mediate group foraging activities, as in the exploratory trails by ants (*e.g.,* Wilson, 1962; Holldobler and Wilson, 1970; Holldobler, 1977; Ritter *et al.,* 1977; Williams *et al.,* 1981) and termites (Stuart, 1970).

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Several species of eusocial insects release an alarm pheromone upon attack by predators to enhance aggregation and aggressive behaviors toward the intruder (*e.g.*, Wilson, 1971; Longhurst *et al.*, 1979; LaMon and Topoff, 1981; Howse, 1984). As far as we know, however, there is only one example in the chemoreception literature of anti-predatory defensive grouping prior to predator detection. In this example, both sexes of the beetle, *Lycus loripes*, were found to aggregate by means of chemical emission from the males (Eisner and Kaftos, 1962). Since *Lycus* releases an offensive odor when attacked, group living presumably aids the individual by reinforcing avoidance behavior in predators.

The present study provides data on chemical attraction leading to the formation of aggregations in a marine decapod crustacean, *Panulirus interruptus* (Randall). Field and laboratory studies have shown that the California spiny lobster forages at night and resides in shelters during daylight hours either alone or in groups (Lindberg, 1955; Roth, 1972). In contrast to the clawed lobsters of the family Nephropidae (Atema and Cobb, 1980), communal den occupancy and aggregation behavior occurs throughout the family Palinuridae (Lindberg, 1955; Fielder, 1965a; Berry, 1971; Berrill, 1975, 1976; Herrnkind *et al.*, 1975; Cobb, 1981), though its mechanism has previously been undetermined. Shelter selection by one individual followed by chemically mediated, conspecific attraction could lead to the formation of these aggregations. In this study, we establish that substances released by spiny lobsters attract conspecifics to shelters. Further evidence shows that both chemical release and attraction are not sex specific and that shelters containing dead conspecifics are avoided. The observed chemical attraction is assigned an anti-predatory function.

MATERIALS AND METHODS

Field experiments

Field trapping experiments were performed to provide correlative information concerning the formation of aggregations by *Panulirus interruptus*. It was the purpose of these experiments to provide capture-frequency data for comparison to theoretical Poisson and negative binomial distributions. The Poisson distribution has been used to test for randomness and independence among captures (Sokol and Rohlf, 1981), while the exponent, k, of the negative binomial distribution measures over-dispersion and aggregation (Taylor *et al.*, 1979). A value of k approaching infinity occurs when capture is random, while k approaches zero as capture becomes increasingly contagious. An iterative process was used in estimating k (Bliss and Fisher, 1953).

A 50 m × 100 m quadrat was established in December, 1978, approximately 150 m offshore in 4–7 m depth over a rocky reef in the More Mesa coastal area of Santa Barbara, California. Unbaited traps, modified to allow both entrance and exit, were placed to simulate heretofore uncolonized shelters. Trap selection by a founding lobster appeared to be based primarily on thigmotaxis. Traps were positioned in a 2 × 4 matrix with adjacent traps separated by 25 m. Traps were set at 0800–1000 h and pulled after 24 h on 10 occasions. Replicate experiments were performed during June, 1979, and again in July, 1980. In these later tests, unbaited traps were positioned at 16 permanently buoyed stations that were located slightly inshore (2–4 m depth) of the quadrat area. All captured animals were tagged before release (Zimmer-Faust and Case, 1982), but no recaptures occurred during these experiments.

Laboratory experiments

Collection and maintenance of animals. Lobsters of 65–102 mm carapace length were captured by trapping or by hand (SCUBA) at More Mesa reef and brought immediately to laboratory holding tanks (1.2 m diameter × 0.6 m depth). Animals were maintained in tanks for two weeks prior to experimentation, with aeration and ambient sea temperature (15–18°C) maintained by a constant seawater flow. A 12:12 D:L (light on: 0800 h) cycle was imposed, starting at least 7 days before tests. Animals were fed mackerel on alternate days and tanks were drained and thoroughly rinsed after each feeding to ensure that residual food odors did not contribute to responses in subsequent experiments.

Test tank. All tests were performed in a large, rectangular fiberglass tank (9.0 m × 2.4 m × 1.0 m). Seawater entered at one end of the tank through regularly spaced holes in a horizontally positioned pipe, and flow (28 $\text{ml} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$) was rendered semi-uniform by a vertically aligned plexiglass baffle system and a horizontal stack of 0.6 m × 1.3 cm PVC pipes. Outflow was established by two 7.2 cm diameter PVC stand pipes symmetrically positioned at the downstream end of the tank so as to maintain a constant water depth of 45 cm. Plexiglass observation windows positioned in the tank along the sides and bottom enabled direct observation of lobster behavior. To assist traction of walking animals, bottom windows were covered with 0.6 cm mesh vexar, and artificial substratum was attached to the bottom surface. A rectangular 0.5 m × 1.0 m PVC sheet was suspended horizontally over the most downstream observational window, to provide a starting point for test animals. Lobsters readily congregated under this overhang when they were introduced during daylight hours, 6 h prior to the initiation of experiments.

Use of traps as shelters. Elliptically shaped (100 cm length × 79 cm width × 30.5 cm height) polyethylene mesh traps (Fathoms Plus Co., San Diego, CA) were used as shelters in these experiments. We considered the traps to provide an adequate shelter stimulus because (1) lobsters readily entered and left traps in laboratory experiments, following modification of trap entrances to produce shallow slope and height (15 cm), and (2) unbaited traps often captured lobsters in field experiments (Zimmer-Faust and Case, 1982, 1983). Trap residencies by lobsters were maintained by choice in the present experiments.

General procedures. Two traps (shelters) were paired side by side, 4.0 m upstream from the PVC overhang. A stimulus was added to one but not to the other trap, to test for its influence on inherent trap attraction. Additional trials were later used to evaluate the relative influences of two competing stimuli, by placing each in opposing traps.

For each test four lobsters were put in the test tank at 1400 h and immediately took up residency under the PVC overhang. The selection of lobsters for each test was at random, except that each was subjected only once to a given stimulus. Stimuli were introduced into traps at 1945 h, just 15 min prior to the onset of the night phase. Final residency patterns were recorded the following morning at first light (0800 h), since observations showed that each lobster retained its residency throughout the day (0800–2000 h). Periodically, we recorded patterns of shelter occupancy during the night to determine when animals first left the PVC overhang area and when they first took up residency in traps. Movement of animals from one trap to the other was assessed by watching marked animals through plexiglass windows using dim red illumination.

We limited experiments to the months of June–December, 1982–1984, to avoid possible complications from reproductive activity. Mating occurs in *P. interruptus* during the January–May interval (Mitchell *et al.*, 1969). Only hard-shelled lobsters were used, and females carrying eggs or spermatophores were excluded.

Experiment A: responses of lobsters to conspecific odors. Tests were first performed to determine whether *Panulirus interruptus* releases substances which attract conspecifics. Either four male or four female lobsters were used as test animals during each trial, and a stimulus animal, either male or female, was isolated in one of two opaque headtanks (30 × 30 × 20 cm) positioned 0.5 m above the test tank. The opacity of the headtanks ensured that visual contact was not made between stimulus and test animals. Continuously flowing seawater was conducted from one headtank (3.0 l/min) to the center of one of the traps, while the opposing headtank conducted seawater into the alternative trap. Both stimulus and test animals were replaced and the position of the stimulus animal was reversed with each successive trial.

Experiment B: responses of lobsters to living and dead conspecifics, living and dead crabs, and prey tissues. Additional tests were performed to determine whether any conspecific attraction in the first experiment resulted from foraging or shelter-related gregarious activities. Attraction of lobsters to carrion placed in shelters was used to assay the possibility that foraging caused aggregations, while attraction only to live conspecifics and not to carrion was taken to indicate that shelter-related behavior was gregarious in nature. We used only male animals in these experiments due to time constraints, and because male and female test animals responded nearly identically to odors presented in the first experiment (see Results, Table II).

Abalone (*Haliotis* spp.) muscle was used as a carrion stimulus, because of its preferred status as food to *P. interruptus* (Carlberg, 1975) and because of its proven ability to attract lobsters in field trapping experiments (Zimmer-Faust and Case, 1982, 1983). Carrions derived from killed lobsters and crabs (*Cancer antennarius*) and from lobster thoraces and abdominal muscle tissues were also tested. Animals were killed just prior (<5 min) to their use in experiments by crushing between the eyestalks after which a dorsal incision was made laterally between the thorax and the tail to expose underlying tissues. Alternatively, some animals were killed for tests by holding on dry ice for 10 minutes. This latter procedure minimized damage to the exoskeleton, while the former maximized release of body and tissue fluids.

In each trial, either carrion or a live animal was placed in a cylindrical, 6-mm mesh vexar container and attached to the center bottom of one trap. The container allowed odor release, while preventing contact between its contents and test animals. An identical empty container in the opposing trap controlled for container volume and surface. Headtanks were not used in these tests, to minimize the dilution of potential feeding attractants. Owing to their fragility, lobster abdominal muscle and abalone muscle (100 g wet tissue) were protected from physical dispersion by being placed in 3-mm mesh vexar bags before being put in containers.

RESULTS

Qualitative field observations

Observations were made of aggregations using SCUBA at More Mesa reef, during April, 1979, and again in June, 1980. Dens were abundant along undersides of large boulders which had become undercut and the sand removed by tidal surge, and also among the rubble of collapsed or fractured rocky reef ledges (Fig. 1). As many as 30 or more lobsters occupied a single den while a nearby (<5 m distant) refuge of seemingly equal quality was present and uninhabited. A total of 16 dens having multiple occupancies of both male and female lobsters, approximately 65–90 mm in carapace length, were marked by buoys and monitored for 5 consecutive days. No attempt was made to tag individuals or to follow the residency patterns of individuals, since such disturbances could have adversely affected communal behavior. Of the total, eight dens had multiple occupancies on each day. The remaining eight dens exhibited a variety

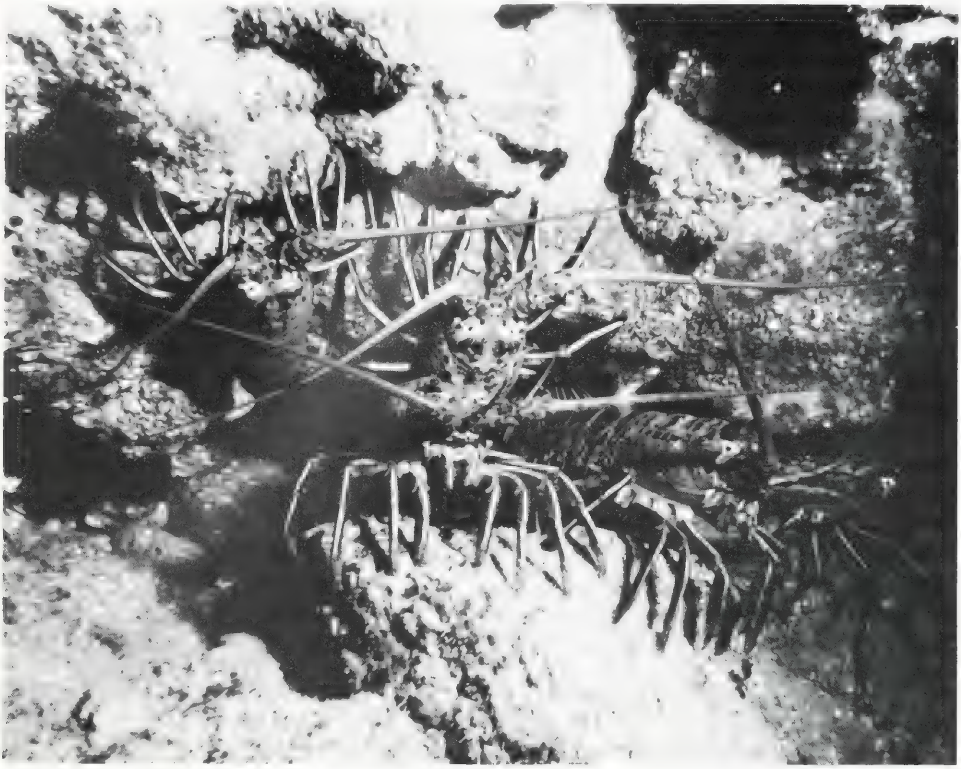

FIGURE 1. Natural habitat of *Panulirus interruptus* showing a den occupied by five animals at More Mesa reef. Though physical characteristics of dens vary considerably, most are typified by having at least two entrances. One entrance is usually large and open, as seen here, and lobsters orient themselves so that their antennae point outwards. The second entrance is generally much smaller and it is often situated to the rear, where it is used by lobsters to escape if a large predator should penetrate from the front. Lobsters in this photograph are of 65–75 mm carapace length. Photograph courtesy of J. R. McCullagh, Marine Science Institute, U. C. Santa Barbara.

of residency patterns, with occupancies occurring over 1–3 day intervals. Dives both by night and by day in April, 1979, revealed that lobsters did not reside in dens at night, even though they did during the day. Our observations show that aggregations of lobsters are naturally associated with shelters and that both emigration and recolonization occur on a nightly basis.

Field experiments

Distributions of lobsters captured in unbaited traps differed significantly from those expected by Poisson distributions (Table I and Chi-square Goodness-of-Fit Tests, Expt. 1: $\chi^2 = 31.76$, d.f. = 3, $P < 0.001$; Expt. 2: $\chi^2 = 17.68$, d.f. = 2, $P < 0.001$; Expt. 3: $\chi^2 = 5.06$, d.f. = 2, $P = 0.07$). There was a strong tendency for Poisson models to overestimate captures of single animals, while they underestimated the likelihood of null and of multiple captures, indicating lack of independence between successive capture events. Observed capture frequency distributions were adequately modelled by the negative binomial distribution, with exponent k assuming values that approached

TABLE I

Frequency of lobster capture in unbaited traps in field

						Capture frequency		
						Expected		
	Number		Size[a] (mm)		Number (lobsters/ trap · day)			
Experiment	Male	Female	x̄ ± SEM	Range		Poisson	Negative binomial[b]	Observed
1 (Dec., 1978)	26	53	69.6 ± 1.2	36–97	0	24.63	46.00	46
					1	29.04	13.93	17
					2	17.04	7.29	9
					3	9.28	4.33	2
					≥4	2.64	8.45	6
2 (May, 1979)	36	34	75.8 ± 1.8	45–121	0	38.82	53.01	53
					1	19.41	4.78	4
					2	4.85	2.18	2
					3	0.81	1.25	2
					≥4	0.11	2.78	3
3 (July, 1980)	7	11	69.7 ± 3.3	46–90	0	64.68	70.00	70
					1	13.75	6.25	7
					2	1.46	2.01	1
					3	1.10	0.82	0
					≥4	0.01	0.92	2

[a] Measured as carapace length.

[b] *k* values were 0.408 for Dec., 1978; 0.110 for May, 1979; 0.154 for July, 1980.

zero (Table I and Chi-square Goodness-of-Fit Tests, Expt. 1: $\chi^2 = 3.04$, d.f. = 3, $P > 0.30$; Expt. 2: $\chi^2 = 0.23$, $P > 0.80$; Expt. 3: $\chi^2 = 0.24$, d.f. = 2, $P > 0.80$). This indicated that capture was highly contagious and over-dispersed. Such over-dispersion was not wholly the result of spatial (trap positioning) or temporal (daily environmental variability) heterogeneity in the probability of capture, since catch was nearly homogeneous at each trap site in all experiments (Chi-square Test-for-Homogeneity: $P \geq 0.10$, for each replicate test) and differences in total daily captures were non-significant ($P > 0.20$) except in the first experiment (Chi-square test: $\chi^2 = 36.62$, d.f. = 9, $P < 0.001$). These results indicate that factors other than habitat and environmental variabilities are in part responsible for observed capture frequencies, with conspecific attraction a likely factor. There were no apparent differences in size frequencies or sex ratios of animals captured in individual traps, as compared to the total sample captures.

Laboratory experiments

Field experiments and observations provided only correlative evidence for conspecific attraction, and laboratory experiments were essential to determine the nature of stimuli underlying the formation of aggregations.

Experiment A. Both sexes of test animals were found to be significantly attracted to traps (shelters) having conspecific effluence introduced from either isolated male or female stimulus animals (Table II). No preferences were exhibited by either sex of test animal for male or for female effluence, when these stimuli were paired in opposing traps. Results show that both male and female lobsters release chemicals attractive to

TABLE II

Trap selection by lobsters in the presence of conspecific odors

Test animal sex	Stimulus		Number of animals residing in traps		Significance[a]	Total number of animals tested
	Test	Alternative	Test	Alternative		
Male	Live male lobster	Empty trap	20	7	$P = 0.047$	32
	Live female lobster	Empty trap	20	8	$P = 0.039$	32
	Live male lobster	Live female lobster	17	13	$P = 0.300$	32
Female	Live female lobster	Empty trap	24	6	$P = 0.016$	32
	Live female lobster	Empty trap	20	6	$P = 0.008$	32
	Live male lobster	Live female lobster	15	13	$P > 0.400$	32

[a] Determined using Wilcoxon Signed Rank Test.

conspecifics and that both males and females possess receptors for, and respond to, attractants. Chemical stimuli were obviously mediating aggregative behavior, even in an absence of visual or tactile cues.

Experiment B. Live lobsters were found to attract test animals to traps, as in the previous experiment; however, abalone muscle was unable to affect trap residency (Table III). Control experiments which paired empty traps or paired traps, each with a live lobster, showed no trap biases to exist and no directional preference was exhibited by test animals.

Dead lobsters with crushed carapaces, excised lobster thoraces, and abdominal muscle were all found significantly repellent to test animals (Table IV). Lobsters killed by dry ice were not as repellent, which would be expected if repellency was dependent on the amount of tissue exposed. Dead lobsters failed to modify the attraction of test animals to live lobsters, when these stimuli were paired in opposing traps, and repellency

TABLE III

Trap selection by lobsters in the presence of live conspecifics and known preferred food

Stimulus		Number of animals residing in traps		Significance[a]	Total number of animals tested
Test	Alternative	Test	Alternative		
Live lobster	Empty trap	50	16	$P = 0.002$	72
Live lobster	Live lobster	16	13	$P > 0.400$	32
Empty trap	Empty trap	15	11	$P > 0.500$	32
Abalone muscle	Empty trap	19	21	$P > 0.500$	40

[a] Determined using Wilcoxon Signed Rank Test.

TABLE IV

Trap selection by lobsters in the presence of dead conspecifics

Stimulus		Number of animals residing in traps			
Test	Alternative	Test	Alternative	Significance[a]	Total number of animals tested
Dead lobster[b]	Empty trap	4	20	$P = 0.008$	32
Dead lobster[b]	Live lobster	3	24	$P = 0.008$	32
Dead lobster[c]	Empty trap	8	16	$P > 0.200$	24
Lobster thorax	Empty trap	8	21	$P = 0.047$	32
Lobster muscle	Empty trap	7	22	$P = 0.039$	32
Lobster thorax	Lobster muscle	11	8	$P = 0.500$	32

[a] Determined using Wilcoxon Signed Rank Test.
[b] Sacrificed using crushed carapace method.
[c] Sacrificed using dry ice method.

was overridden to some extent by a lack of suitable refuge. This last result was demonstrated by pairing lobster thoraces against lobster abdominal muscle, and finding that 60% of all tested animals still chose to reside in traps. Neither living nor dead crabs were found to influence shelter residency by lobsters (Table V).

Monitoring of shelter residency. Lobsters moved from beneath the PVC overhang (starting shelter) and began entering traps within 30 min after the onset of darkness (Fig. 2). Marked animals moved freely between traps and residencies were not established during an initial 3.5 h period. Although lobsters did not aggregate in traps during this period, they did by the following morning. This was demonstrated by observing shelter residency patterns at 2330 h and again at 0800 h, for a total of 24 trials (Table VI). Observed data were compared to those values predicted for random assortment by a binomial distribution $(p + q)^n$, where exponent n was the total number of animals residing in traps at each observation time for a given trial, and p and q were probabilities of capture for each trap ($p = q = 0.5$). The case where only one animal resided in a trap was not evaluated, because it neither reflected aggregation nor randomness. Differences in values expected for trap residencies at 2330 h and 0800 h were largely a result of different distributions of n.

TABLE V

Trap selection by lobsters in the presence of a sympatric crab

Stimulus		Number of animals residing in traps			
Test	Alternative	Test	Alternative	Significance[a]	Total number of animals tested
Live crab	Empty trap	23	24	$P > 0.500$	56
Live crab	Live lobster	7	22	$P = 0.039$	32
Dead crab[b]	Empty trap	12	12	$P > 0.500$	24
Dead crab[c]	Empty trap	9	7	$P > 0.400$	16

[a] Determined using Wilcoxon Signed Pair Test.
[b] Sacrificed using crushed carapace method.
[c] Sacrificed using dry ice method.

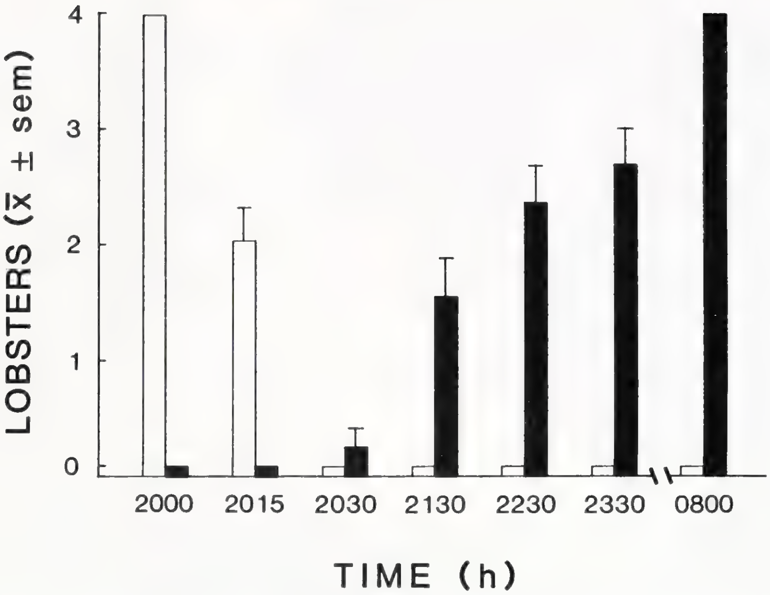

FIGURE 2. Number of lobsters ($\bar{x}$ ± SEM) residing under the PVC overhang area (□), and the number entering traps (■) during a 3.5 h observational period following onset of darkness (2000–2330 h) and again the following morning (0800 h). All animals leave the overhang area within 30 min, but not all animals enter traps within the initial 3.5 h period. A total of 10 trials were performed with four animals per trial.

DISCUSSION

Our results indicate that chemical attraction facilitates conspecific social interactions in the marine crustacean, *Panulirus interruptus*. Specifically, we find that this animal is highly gregarious and that shelter selection influenced by chemical attraction may be the key to explaining the multiple resident denning commonly observed in members of this family. It has been questioned whether communal denning in palinurids is of

TABLE VI

Temporal influence on residency in traps[a]

	Assortment in paired traps[b]			Chi-square test		
Time of observation	4–0 3–0 2–0	3–1 2–1	2–2 1–1	χ^2 value	d.f.	Sig. (*P*)
Night (2330 h)						
Expected by chance	6.88	10.50	4.62	3.81	2	>0.10
Observed	9	6	7			
Day (0800 h)						
Expected by chance	3.00	12.00	9.00	47.19	2	≪0.001
Observed	14	6	4			

[a] n = 24 trials.

[b] See text for explanation.

social origin or is simply a matter of limited availability of shelter (Cobb, 1981). Our results indicate that residency patterns in *P. interruptus* arise in part from non-random habitat colonization and from cohabitation which occurs by choice.

Chemicals mediating cohabitation were not sex specific in either their emission or reception. Because laboratory experiments were performed during non-mating periods, aggregative formation was clearly not associated with reproduction. Abalone muscle, a potent feeding attractant to lobsters, and freshly killed crab both failed to influence shelter selection. Consequently, aggregation formation cannot be attributed to foraging or feeding. This leads us to believe that aggregations are probably antipredatory in function. It has been observed that group residency in the Western Australian lobster, *Panulirus cygnus,* reduces the penetrability of a den to large mobile predators (Cobb, 1981). We have made similar observations, witnessing *P. interruptus* to position itself so that it points its robust spinose antennae outwards from the burrow (Fig. 1). Cohabitating animals collectively wave their antennae to fend off predatory fishes and divers. The repellency of dead conspecifics would appear to facilitate defense and predator avoidance because it can signal the presence of an active, nearby predator (Atema and Stenzler, 1977) or indicate an otherwise sub-optimal refuge. It is assumed that natural selection has favored those animals which avoid diurnally active predators, since *Panulirus* forages only at night and since many, but not all, identified predators of *Panulirus* are diurnally active fishes. These predators include the California sheephead (*Pimelometopon pulchrum*), the kelp bass (*Paralabrax nebulifer*), and the cabezon (*Scorpaenichthys marmoratus*), among others (Lindberg, 1955).

Of particular interest is our finding that both excised lobster thorax and abdominal muscle are repellent to live lobsters. This means that repellency cannot simply be attributed to the release of metabolites concentrated in thorax tissues. The fact that dead crab fails to influence shelter related behavior of *P. interruptus* further suggests that substances mediating both conspecific repellency and attraction may be species-specific. Such species-specific repulsion to dead conspecifics has been reported for at least two other decapods, the lobster, *Panulirus cygnus* (Hancock, 1974), and the crab, *Cancer pagurus* (Chapman and Smith, 1979). Alarm pheromones are known to be released by injured sea anemones (Howe and Sheikh, 1975), by injured gastropod snails, (Snyder, 1966; Atema and Burd, 1975; Atema and Stenzler, 1977; Stenzler and Atema, 1977), by injured sea urchins (Snyder and Snyder, 1970; Mann *et al.*, 1984), as well as by earthworms (Ressler *et al.*, 1968), insects (*e.g.*, Maschwitz, 1964; Wilson, 1971), and aquatic and terrestrial vertebrates (*e.g.*, Muller-Velten, 1966; Reutter and Pfeiffer, 1973; Muller-Schwarze *et al.*, 1984).

Our field and laboratory observations show that aggregations of *Panulirus* form overnight. Supportive evidence comes from underwater video observations conducted during July and August, 1983, at Big Fisherman Cove, Santa Catalina Island, California. Departure from the den occurred shortly after sunset (Tyre *et al.*, unpub. data), a pattern which is repeated in laboratory tests. Lobsters were also found to return to dens in greatest numbers shortly before sunrise, a result which again is in agreement with our laboratory observation that lobsters aggregate by morning but not during the initial period following darkness onset. Changes in predisposition toward aggregation could be attributed to variable rates of chemical emission, variable receptor sensitivities, or to higher order behavioral programs or hormonal control. Whether such predisposition is influenced by ambient light intensity or by a circadian rhythm cannot presently be distinguished. It has been shown for the lobsters, *Panulirus argus, Nephrops norvegicus,* and *Jasus novahollandiae,* that locomotory activity varies according to light intensity and to photoperiod, but such activity is also under circadian control (Sutcliffe, 1956; Fielder, 1965b; Kanciruk and Herrnkind, 1973; Arechiga and Atkinson, 1975).

Nocturnal foraging is readily initated by *Panulirus interruptus* (Lindberg, 1955; Winget, 1969), yet this may not be the only factor motivating departure from dens and search. Shelter seeking as described in this study could, by itself, be a distinctive and separate type of search made necessary because of both long-term seasonal and short-term catastrophic events which constantly change substrate topography, thereby creating, destroying, and modifying refuges. Our field experiments using unbaited traps demonstrate that lobsters colonize novel and previously unoccupied habitats, and nocturnal den switching without associated foraging activity has been observed in *P. argus* (Herrnkind *et al.*, 1975).

We have not yet tested the hypothesis that cohabitation truly reduces vulnerability of individual lobsters to predation. There are two principal mechanisms proposed for group living that account for selective forces which could act to reduce predation, namely, (1) individuals position themselves among cohorts to reduce their own risk of being eaten (selfish herd) (Williams, 1964; Hamilton, 1971; Milinski, 1977), and (2) greater prey vigilance reduces prey response time to predatory attack (Pulliam, 1973; Treisman, 1975a; Treherne and Foster, 1981). Because *Panulirus interruptus* exhibits dominance (Roth, 1972), as well as gregariousness, in its shelter-related behavior it would seem that this animal may be useful in exploring problems of group size, geometry, and composition, as these factors affect predator detection and prey selection by predators. Such experiments are now being conducted in our laboratory.

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PLANKTOTROPHY BY THE LECITHOTROPHIC LARVAE OF A NUDIBRANCH, *PHESTILLA SIBOGAE* (GASTROPODA)

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ABSTRACT

Veliger larvae of the coral-eating nudibranch *Phestilla sibogae* can metamorphose 3–4 days after hatching in the absence of any external food source. If deprived of a settlement stimulus, starved larvae survived as long as 30 days, but had lost metamorphic competence several days before starvation death. Fed larvae survived more than 42 days and retained metamorphic competence for as long as 42 days. Feeding by these potentially lecithotrophic larvae extended the duration of survivorship by at least 28% and the duration of the competent period by as much as 90%. There is evidence from light and electron microscopy for both digestion and uptake of phytoplankton in the larval gut. The ability to extend a brief lecithotrophic larval existence by planktotrophy may explain the wide spread distribution of this species in the tropical Indo-Pacific Ocean.

INTRODUCTION

The planktic larvae of many marine invertebrates complete metamorphosis without feeding (*e.g.*, Mortensen, 1921; Graves, 1932; Thorson, 1946). These larvae utilize energy reserves provided as yolk stored in the egg during oogenesis and are termed "lecithotrophic." In fact, the operational definition for lecithotrophy is the ability to metamorphose without ingestion of visible external food (the potential reliance of presumed lecithotrophic larvae on dissolved nutrients has been little investigated). Lecithotrophic larvae are often large and devoid of a mouth and other feeding structures seen in their planktotrophic relatives; phyla as diverse as the Echinodermata and Bryozoa provide good examples. However, it has been noted in other groups that larvae which have been observed to complete metamorphosis in the absence of an external food supply possess all of the apparent structures for planktotrophy. A common, though little tested assumption engendered by observations of metamorphosis in larvae that have not fed is that such "lecithotrophic" larvae cannot or at least do not ingest and assimilate planktonic food (*e.g.*, Obrebski, 1979). Examples of feeding by potentially lecithotrophic larvae have been reported in the literature (Thorson, 1946; Thompson, 1958, 1976), but they have been considered to have little importance, and, as a result, scant attention has been given to experimental investigation of the importance of feeding to such facultatively planktotrophic larvae. They are capable of completing metamorphosis without reliance on external food sources, but feeding may extend their larval lifespan and produce variation in age at metamorphosis (Hadfield, 1963).

There are observations suggesting that investigation of feeding by functionally lecithotrophic larvae might provide insight into the larval ecology of a number of

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invertebrate species. Thompson (1958), in his studies on the development and metamorphosis of lecithotrophic larvae of the dorid nudibranch *Adalaria proxima,* found that diatoms were ingested by the larvae and were incorporated into cells of the left digestive diverticulum by endocytosis. Knowlton (1973) noted that the lecithotrophic larvae of the decapod *Alpheus heterochaelis,* ". . . apparently feed despite an amount of yolk in their stomachs sufficient to sustain them through the larval phase." Hadfield (1972) observed that the functionally lecithotrophic larva of a nudibranch, *Phestilla sibogae,* could ingest phytoplankton, and Hadfield and Switzer-Dunlap (1984) reported that larval survival was extended for several weeks when single-celled algae were present in cultures.

A number of authors have noted certain inherent disadvantages in a larval existence limited to lecithotrophy (*e.g.,* Thorson, 1946; Mileikovsky, 1971; Chia, 1974; Crisp, 1976; Strathmann, 1978). The planktic period for such larvae is restricted to the length of time that their metabolism can be supported by energy reserves stored in the egg. As a result, their ability to survive in the plankton, in the absence of suitable settlement substrata, is substantially less than that of many species with planktotrophic larvae. Nevertheless, there is evidence of extended metamorphic competence periods for some nonfeeding larvae (*e.g.,* Birkeland *et al.,* 1971). Shuto (1974), Scheltema (1977, 1978), Jablonski and Lutz (1983), and others have suggested that the lecithotrophic limitation on dispersal could significantly affect a species' survival over geological time.

The studies described here present morphological evidence that functionally lecithotrophic larvae of the nudibranch *Phestilla sibogae* can feed and experimental data on the extent to which fed larvae exceed starved larvae in duration of survivorship. Metamorphic success in older fed and starved larvae is also explored.

MATERIALS AND METHODS

Adults of *Phestilla sibogae* were collected from Kaneohe Bay, Oahu, Hawaii and maintained in flow-through sea water tables. Egg masses from these animals were incubated in mesh containers suspended in flowing sea water, and larvae were artificially hatched by ripping the egg masses apart with fine forceps about one day prior to the date when spontaneous hatching would have occurred; this was the seventh day after egg deposition in most cultures. Newly hatched larvae used for mass determinations were cultured as described by Switzer-Dunlap and Hadfield (1981). Larvae were maintained in either 0.45 μm Millipore-filtered (Cat No. AP2504700) sea water and no food, or in Millipore-prefiltered sea water containing the unicellular alga *Pavlova lutheri* at a concentration of 10^4 cells/ml. Temperatures ranged between 24 and 27°C. Culture water was changed every three days, and an amount of cultured algae sufficient to give a concentration of 10^4 cells/ml was added to the fed cultures after each change. Culture A consisted of unfed larvae that were sampled after 3, 9, 15, 21, and 27 days. A second pair of cultures, one starved (B-1) and one fed (B-2), was set up from a single batch of newly hatched larvae. Larvae in these cultures were sampled at hatching and after 20 days of starvation or feeding. Larvae were counted into small, pre-weighed aluminum pans for weighing; for Culture A, 10 pans each containing 10–20 larvae were weighed for each early sample, but the number declined to as few as 2 larvae/pan (10 pans) for later samples as larval numbers decreased due to mortality. For B cultures, 10 pans (each containing 10 larvae) were weighed on each sampling date.

Since the shell of the larva of *Phestilla sibogae* does not grow (Bonar and Hadfield, 1974; Harris, 1975), the shell mass for the larvae in the sampled cultures was assumed to be equal to that determined from a single weighing of 10 empty, dry, larval shells collected from Culture A (0.52 μg/shell). This shell mass value was compared to that of two samples of newly hatched larvae from different egg masses that were dried to constant mass at 80°C, weighed, ashed at 500°C for 3 hours, and re-weighed (0.54–0.57 μg/shell). Since the larval shell masses determined by these two methods were similar, tissue mass for larvae from starved and fed cultures was calculated by subtracting the average mass per shell of dried shells (0.52 μg) from the average total mass of a larva at each sampling date. All mass determinations were made on a Cahn 25 Automatic Electrobalance. Significance of the observed changes in tissue masses of fed and starved larvae was determined with a Wilcoxon-Mann-Whitney rank-sum test (Snedecor and Cochran, 1967).

Differential survival of fed and starved larvae was investigated under a variety of culture conditions (Cultures 1–10). A series of paired groups of cultures of fed and starved larvae was set up as described above. Fed cultures were provided with algal cells at concentrations of 10^4 or 10^5 per milliliter; the phytoplankter *Pavlova lutheri* was provided alone (Cultures 5–9) or mixed with *Phaeodactylum tricornutum* (Culture 3), *Isochrysis galbana* (Cultures 1 and 2), or the so-called "Tahitian strain" of *Isochrysis* (Culture 10). Other conditions were: larval concentrations of 0.5, 0.8, 1.0 or 1.5 larva/ml; illumination as constant light, constant dark, or ambient light; these variables did not significantly affect larval longevity. The antibiotics penicillin G (60 μg/ml) and streptomycin sulfate (50 μg/ml) were added to each culture; temperatures varied between 24 and 27°C. The number of living larvae in each culture was determined every 2–3 days until it had decreased to near zero. For six similar cultures, the average difference in duration of survivorship between fed and starved larvae was determined by the t-test for matched pairs (Schafler, 1979, pp. 94–97). Significance of the difference in survival duration between the longest-surviving fed and longest-surviving starved larvae for all sets of culture conditions was determined with Wilcoxon's Signed-Ranks Test (Sokal and Rohlf, 1981, pp. 448–449). In some of the culture sets, larvae were tested for competence to metamorphose near the maximum duration of survival by exposing them to small pieces of coral or to a lyophilized, distilled-water extract of coral dissolved in sea water (Hadfield, 1977).

Larvae were fixed for light and electron microscopy in order to examine the structure of the digestive organs. Prior to fixation, larvae were relaxed for five minutes in a mixture of one part sea water saturated with chlorobutanol to three parts filtered sea water in a −20°C freezer. Following this treatment the larvae were fixed, decalcified, and embedded as described by Bonar and Hadfield (1974). After decalcification, larvae were rinsed three times in 2.5% sodium bicarbonate at pH 7.2 before proceeding to secondary fixation. Sections cut for light microscopy were stained with Richardson's stain (Richardson *et al.*, 1960). Ultrathin sections were stained with a saturated solution of uranyl acetate in 50% methanol at 60°C for 15–20 minutes (Bickell *et al.*, 1981) and lead citrate for 10–20 minutes at room temperature (Venable and Coggeshall, 1965). Stained, ultrathin sections were examined and photographed in a Philips 201 electron microscope.

RESULTS

Newly hatched larvae of *Phestilla sibogae* are similar in structure and appearance (Bonar and Hadfield, 1974) to obligately planktotrophic nudibranch veligers that are near metamorphic competence (Bickell, 1978; Bickell and Chia, 1979; Bickell *et al.*, 1981; Bickell and Kempf, 1983). Although they are functionally lecithotrophic (*i.e.*, they can metamorphose without feeding), newly hatched larvae of *P. sibogae* ingested unicellular algae when they were available. Shortly after they began feeding, the left digestive diverticulum of these larvae became yellow-brown, a coloration similar to that observed in fed, planktotrophic larvae of other opisthobranch species (Switzer-Dunlap and Hadfield, 1977; Kempf and Willows, 1977). In unfed larvae the left diverticulum remained unpigmented. It was observed that larvae trap and ingest single-celled algae with the velar ciliary apparatus, a structure as well developed in the larvae of *P. sibogae* as in veligers of obligately planktotrophic nudibranchs (Thompson, 1959; Strathmann and Leise, 1979). Histological and ultrastructural examination of larvae of *P. sibogae* revealed a complete digestive system including esophagus, stomach, left and right digestive diverticula, intestine, and anus (Fig. 1A). The stomach wall is composed of three major structural areas identical to those previously described histologically (Thompson, 1959; Bickell, 1978; Bickell and Chia, 1979), and ultrastructurally (Bickell *et al.*, 1981; Kempf, 1982) in the planktotrophic larvae of various nudibranch species. These are the densely ciliated band of the dorsal stomach, the gastric shield, and a sparsely ciliated area or vestibule of the ventral stomach (Fig. 1B).

In fed larvae, the left digestive diverticulum was packed with ingested algal cells. Many active digestive cells were present in the diverticulum wall (Fig. 1A). The lumenal surface of these cells bore numerous microvilli and sparse cilia (Fig. 1C, D). Pinocytotic vesicles were forming between some of the microvilli (Fig. 1C). The cytoplasm contained an active lysosomal system, including pinosomes, phagosomes, and multiple large heterophagosomes (Fig. 1D). Numerous mitochondria, basal lipid deposits, and a basal nucleus were also present (Fig. 1D). Identifiable, phagocytosed algal cells were observed in some of the digestive cells (Fig. 1E).

After periods of three weeks or longer, the volume of the visceral mass of fed larvae appeared as large or larger than at hatching, while the visceral mass of starved larvae had obviously shrunk to much smaller proportions than were present at hatching (Fig. 2). These qualitative observations were confirmed by comparing the mean masses of larvae that had been fed or starved for 20 days to each other and to their mass at hatching. There was a slight (ca. 5%), but significant ($P = 0.004$), increase in the tissue mass of larvae that had fed; starved larvae had lost about 40% of their hatching tissue mass in the same period (Fig. 3).

Larvae of *Phestilla sibogae* survived and retained competence to metamorphose for as long as 42 days after hatching when fed the unicellular alga *Pavlova lutheri* alone or in combination with the Tahitian strain of *Isochrysis* (temp. 24–25°C). Starved larvae were observed to survive a maximum of 30 days, but among those tested, metamorphic competence was never retained longer than 22 days (Table I). Temperature affected the results, and in experiments conducted when Hawaiian sea water temperatures are at their highest (September, 27.0–27.5°C), unfed larvae perished by about 17 days post-hatching, while fed larvae were still healthy and capable of metamorphosis at 30 days when the experiment was terminated. The oldest starved larvae in any experiment were degenerate and not capable of metamorphosis (Fig. 2D). Light regime had no apparent effect on larval longevity whether fed or starved.

The data on larval longevity were not all obtained in identical experiments and have thus been examined in several ways. There are also two aspects to larval survivorship to be considered: the total time that any larva remained alive in a culture and the ability of older larvae to successfully metamorphose. In Table II, survivorship alone is analyzed for six experiments where feeding was the same (10^4 cells/ml *Pavlova lutheri*); since each experiment consisted of paired cultures, one fed and one starved, established from single sibling larval groups, the differences in duration of survival were examined by the t-test for matched pairs. Mean survivorship for starved larvae was 24.3 days, for fed larvae 31.1 days. The mean difference in survivorship of 6.8 days is statistically significant ($P < 0.001$), and while not appearing to be large, is an increase of 28% in the duration of larval life. When all data are examined, culture sets

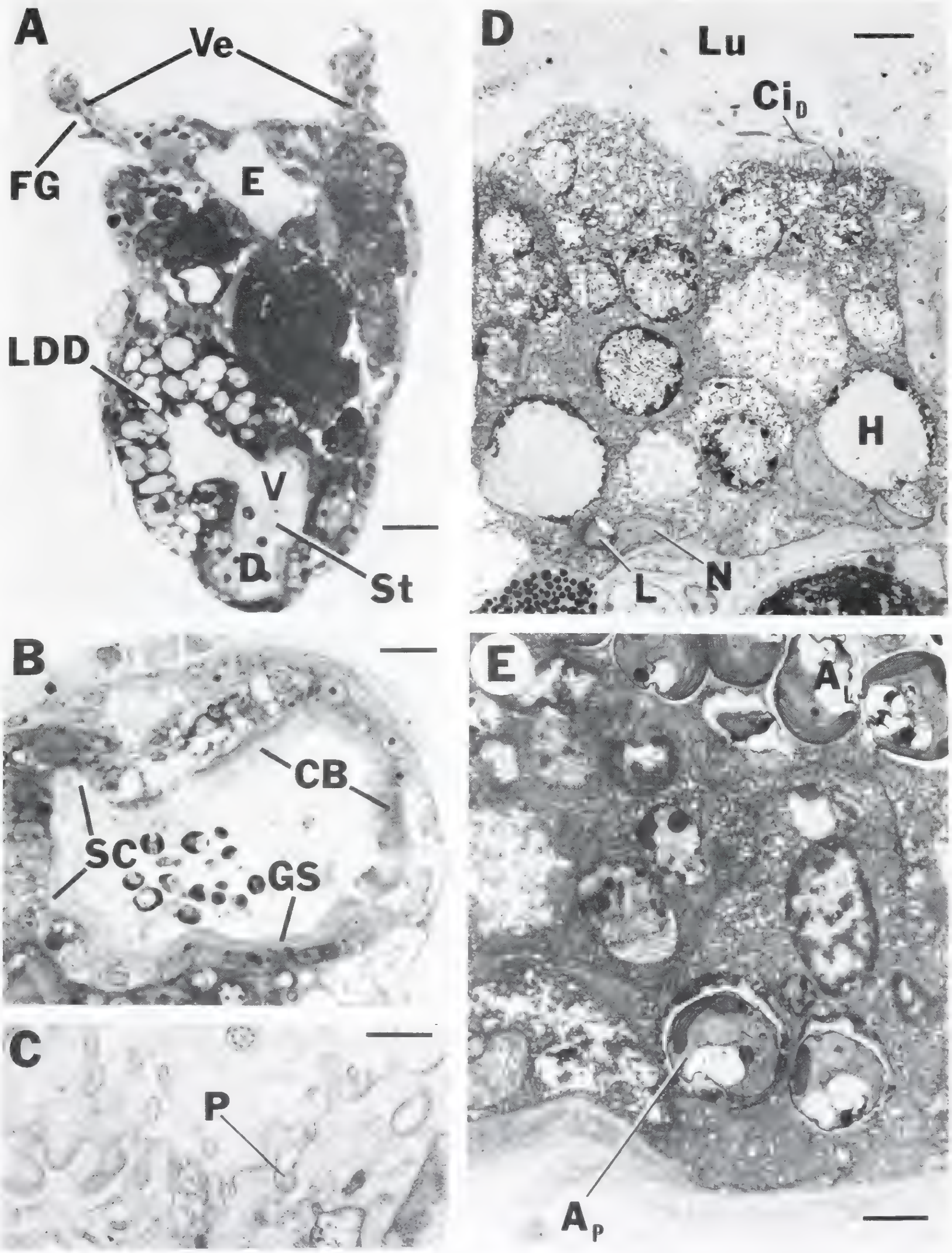

FIGURE 1. A. Frontal section of a larva of *Phestilla sibogae* that had fed on *Pavlova lutheri* for 19 days; scale bar: 20 μm. B. Larval stomach of *P. sibogae;* scale bar: 10 μm. C. Pinocytosis in cells of the lumenal border of the left digestive diverticulum; scale bar: 0.5 μm. D. Active digestive cells of the left digestive diverticulum; scale bar: 3 μm. E. Phagocytosed algal cells within a digestive cell of the left digestive diverticulum; scale bar: 2 μm. A_L, algal cells in lumen of left digestive diverticulum; A_p, phagocytosed algal cell; CB, densely ciliated band of the stomach; Ci_D, cilium of a digestive cell; D, dorsal stomach; E, esophagus; FG, food groove of the velum; GS, gastric shield; H, heterophagosome; L, lipid droplet (partially extracted); LDD, left digestive diverticulum; Lu, lumen of the left digestive diverticulum; N, nucleus of digestive cell; P, pinocytosis; SC, sparsely ciliated area of stomach; St, stomach; V, ventral stomach; Ve, velum.

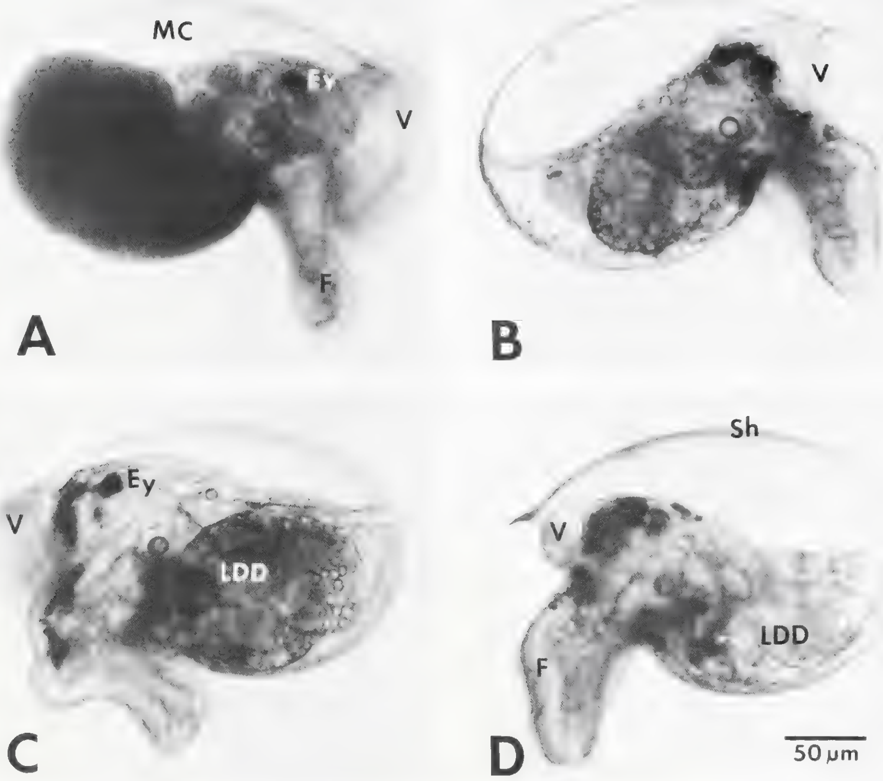

FIGURE 2. A. Newly hatched larva of *Phestilla sibogae;* high tissue density is due to yolk. B. Larva starved for 16 days. C. Larva after feeding for 21 days. D. Starved larva 21 days after hatching, larval organs are in regression, particularly the velum. Ey, eye; F, larval foot; LDD, left digestive diverticulum; MC, mantle cavity; Sh, larval shell; V, velum (out of focus in A).

of fed larvae retained survivors an average of 10 ± 4.5 days (n = 9) longer than starved larvae ($P < 0.01$). The maximum difference seen between starved and fed cultures in ages of larvae competent to metamorphose was 20 days (22 days for starved, 42 days for fed; see Table I); thus feeding allowed a maximum increase of 90% in functional larval lifespan.

DISCUSSION

While not previously investigated experimentally, facultative planktotrophy has been reported under a variety of names. As is so often the case, one finds in Thorson's (1946, 1950) summaries numerous references to such larvae which he labeled, "Planktotrophic larvae with a short pelagic life." Thorson characterized these larvae as briefly planktic (a few hours to a few days), well developed at hatching, and changing little or not at all before settling, and, while capable of feeding in the plankton, usually able to settle without feeding. Thorson included in this category a series of polychaetes, a few prosobranchs, and still fewer opisthobranchs. His remarks were not based on laboratory rearing studies, but rather on the work of others combined with his own

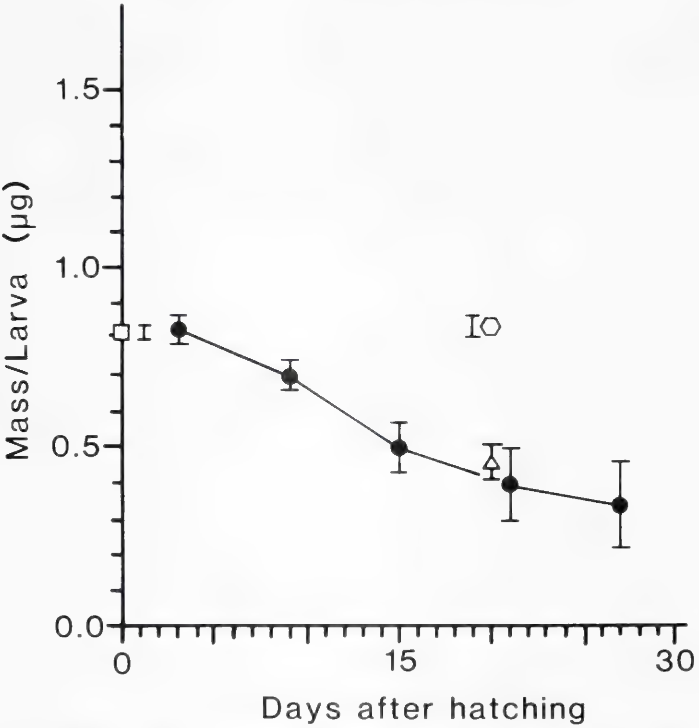

FIGURE 3. Changes in mass of fed and starved larvae of *Phestilla sibogae.* ● —mean tissue mass of starved larvae of Culture A; □ —mean tissue mass of newly hatched larvae of Cultures B-1 and B-2; ○ —mean tissue mass of fed larvae after 20 days, Culture B-2; △ —mean tissue mass of starved larvae after 20 days, Culture B-1. Error bars equal 1 S.D.

observations of larvae that had similar sizes and degrees of development at hatching and settlement. Thorson considered the major role of such larvae to be in dispersal and habitat selection, and feeding to have only secondary importance. He also noted (1946, p. 435) that time spent in the plankton by such larvae ". . . may probably

TABLE I

Metamorphic competence in fed and starved larvae of Phestilla sibogae *after varying periods of larval culture*

	Starved larvae			Fed larvae		
Culture[a]	Days (post-hatch)	No. tested	% metam.	Days (post-hatch)	No. tested	% metam.
1	24	0	—	24	4	25
2	23	10	0	42	18	28
3	22	10	80	42	19	63
7	—	—	—	28	7	0
9	28	2	0	36	5	60
10	21	4	0	29	10	40

[a] Larvae in Cultures 4, 5, 6, and 8 were not tested for metamorphic competence.

TABLE II

Comparison of longevity of larvae between matched pairs of fed and starved cultures

Culture	A max. life starved larvae (days)	B max. life fed larvae (days)	(B–A)
4	17	24	+7
5	30	40	+10
6	25	30	+5
7	24	28	+4
8	22	29	+7
9	28	36	+8
$\bar{X} \pm$ S.D.	24.3 ± 4.6	31.1 ± 5.8	6.8 ± 2.1**

** t-test for differences between matched pairs analysis (Schafler, 1979, pp. 94–97), $t = 7.81$, d.f. $= 5$; $P \ll 0.001$.

All cultures fed *Pavlova lutheri* at 10^4 cells/ml; temp. 25–27°C. Ages are given in days, post-hatching.

vary . . ." We agree with most of these comments, but must add the observation that the onset of metamorphic competence during the pelagic phase represents a significant physiological, although often not morphologically prominent, change in the organization of the larva.

Subsequent to Thorson's studies other species have been cited, either clearly or less so, as falling into a category of facultative planktotrophs. For a number of these, the original observers did not say that feeding was optional (*e.g.*, Allen, 1961, for *Pandora inaequalis;* Kessel, 1964, for *Acmaea testudinalis*), and it is the interpretation of later authors (Ocklemann, 1965; Jablonski and Lutz, 1983) that feeding in these cases was facultative mainly because the planktic feeding period is very brief. Clarification is obviously needed. Yet, facultative planktotrophy seems clearly established for some molluscs, including the nudibranch *Adalaria proxima* (Thompson, 1958), the prosobranch *Conus pennaceus* (Perron, 1981), and the bivalve *Codakia orbicularis* (Alatalo *et al.*, 1984). In both *A. proxima* and *C. pennaceus*, the situation is quite similar to the observations reported here for *Phestilla sibogae*. However, Thompson did not attempt to examine the potential extension of larval life in *A. proxima* while they were feeding, and neither author examined the structure of the digestive system of feeding larvae. Perron (1981) reported, as we do here, a noticeable change in the color of the digestive gland of feeding larvae. R. B. Emlet (University of Washington; pers. comm.) noted that fed, functionally lecithotrophic larvae of the echinoderm *Clypeaster rosaceus* metamorphose at a larger size than those not exposed to phytoplankton, and that resulting juveniles grow faster and may show greater survival than juveniles resulting from metamorphosis of starved larvae. Feeding did not appear to greatly extend the duration of the larval period in *C. rosaceus*.

Conus pennaceus is capable of metamorphosis upon hatching, and has only the simple prerequisite of a biologically filmed surface to stimulate settlement. However, when kept in stringently cleaned glassware (a situation that may approximate conditions when larvae are swept out to sea), unfed larvae survived as long as 29 days, and fed larvae up to 37 days (Perron, 1981), amounting to a 27% increase in larval longevity with feeding, a figure comparable to that observed in the present studies of *P. sibogae*. A major difference between the two species lies in the requirement for the presence of its adult prey, corals of the genus *Porites*, to induce settlement and metamorphosis in *P. sibogae* (Hadfield, 1977).

The term "facultative planktotrophy" was invoked by Chia (1974) to describe the case in which larvae capable of metamorphosing without feeding are observed to ingest food during the larval stage. The term implies that feeding is not obligate. In the early part of their planktic existence, competent larvae of *Phestilla sibogae* certainly appear to fit this designation. We have successfully reared thousands of these animals through full life cycles without ever feeding the larvae (Hadfield, 1984). However, as the competent larvae age without metamorphosing, their ability to survive without feeding declines, and their capacity to complete metamorphosis degenerates well before their starvation death. Feeding in these older larvae is clearly no longer facultative, but obligate.

It has been well argued that the importance of planktotrophy lies not in the time made available for larvae to travel great distances, but rather in the access it provides to an extra-embryonic food source and thus the potential to settle at considerably greater sizes than are possible with larvae dependent on egg yolk alone (a factor frequently "adjusted" by increasing egg size) (Spight, 1976; Strathmann, 1980). It may well be that facultative planktotrophy provides for considerable variance in size at settlement, and, consequently, for differential survival success in different habitats. Feeding by facultatively planktotrophic larvae could provide for greater energy reserves at metamorphosis and concomitantly greater post-metamorphic survival (Crisp, 1976). By permitting extension in the planktic life of lecithotrophic larvae, facultative planktotrophy almost certainly allows additional time for substrate selection in larvae that are widely scattered over a heterogeneous ocean bottom. This possibility, born out by our laboratory studies of larvae of *Phestilla sibogae,* was suggested by Todd (1981).

It might be questioned whether planktotrophy has any natural significance to a species such as *Phestilla sibogae* because its larvae will normally be released into a habitat crowded with suitable settlement substrata. Three observations are germane to this question: (1) during the two- to four-day precompetent period larvae may be carried far from the boundaries of a coral reef; (2) *P. sibogae* is a species with great geographic distribution, spanning most of the tropical Pacific (pers. obs.) and Indian Oceans (if the synonomy of Rudman, 1981, is correct), a distribution far too great to explain on the basis of a planktic larval period of a few days only; and (3) *P. sibogae* feeds only on massive stony corals of the genus *Porites,* effectively precluding the possibility of dispersal by adult rafting.

Zinsmeister and Emerson (1979) have charted the most likely route of dispersal of shallow water benthic invertebrates to Hawaii; it crosses about 5400 km of open sea. Kempf (1981) noted that such dispersal could take as long as 11 weeks between Japan and the western-most islands of the Hawaiian chain. Such transport must not only have occurred in the colonization of the islands, but must still be occurring frequently enough to inhibit endemic speciation in species such as *P. sibogae.* Similar arguments concerned with oceanic transport have been made by Edmunds (1977) in examining the relationships of the opisthobranch fauna of Ghana and by Scheltema and Williams (1983) for tropical Pacific prosobranchs. The importance of larval longevity to the ecology and evolution of shallow-water, benthic, marine-invertebrate faunas need not lie in a high frequency of dispersal between two distant populations, but rather in the absolute probability of such dispersal occurring in relatively brief geological time intervals (Scheltema, 1978). Immigration can be a rare event and yet sufficient to offset the speciation tendencies of genetic drift or natural selection in relatively isolated populations (Crow and Kimura, 1970, pp. 268–270; Lewontin, 1974, p. 213).

It is evident that larvae of *P. sibogae* retain the hereditary capacity for planktotrophy, while also clearly demonstrating the ability to complete the larval stage without feeding. As such, they appear to represent a transitional stage between planktotrophic and lecithotrophic life histories, retaining advantages of each. Is five to six weeks the limit for planktotrophic larval survival in *Phestilla sibogae?* Probably not. Recent observations with improved culture chambers (circulating water) and better food organisms (such as the Tahitian strain of *Isochrysis*) suggest that these larvae may indeed live much longer while in energetic and developmental stasis (unpub. pers. obs.). For these reasons, we feel that larval transport can readily explain the broad distribution of this species.

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EMBRYONIC DEVELOPMENT OF THE BRITTLE-STAR *AMPHIPHOLIS KOCHII* IN LABORATORY CULTURE

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ABSTRACT

The embryonic development of the brittle-star *Amphipholis kochii,* from fertilization through metamorphosis, was observed in a laboratory culture. Oocytes from spawning induced by a sudden change of sea water temperature remain in the first meiotic metaphase until fertilization. The unfertilized egg, about 90 μm in diameter, is opaque, brownish red, and homolecithal. The fertilization membrane of the egg is transparent and non-sticky, and the translucent (7–8 μm) hyaline layer is thick. Cleavage is holoblastic and equal. The blastomeres usually are irregularly arranged, although some eggs show the regular radial cleavage. The archenteron is formed by an invagination of the endodermal cells. The developing larval spicules first take a tetraradiate form, unlike the triradiate forms of most other ophioplutei and echinoplutei. The fully grown ophiopluteus has eight arms and a very simple skeletal system. The left posterior coelom is divided into a hydrocoel and a somatocoel. The anterior part of the larva and the larval arms, except for the postero-lateral arms, shrink and degenerate by the onset of metamorphosis.

INTRODUCTION

The ophiuroids have various developmental patterns, and planktotrophic development with a free-living ophiopluteus is typical (Hyman, 1955; Hendler, 1975). However, recent reports on ophiuroid development have been concerned with the pattern known as abbreviated development (Fenaux, 1963, 1969; Patent, 1970; Stancyk, 1973; Hendler, 1977, 1978; Mladenov, 1979; Oguro *et al.*, 1982). Observations made on development of the ophiopluteus larva are scarce, despite the fact that many ophioplutei have been found by means of plankton-hauls (*e.g.*, Mortensen, 1913, 1920, 1921, 1931, 1937, 1938). To date, detailed studies on ophiopluteus development have been confined to the three species *Ophiothrix fragilis* (MacBride, 1907), *Ophiocoma nigra* (Narasimhamurti, 1933), and *Ophiopholis aculeata* (Olsen, 1942). In addition, reports on the early developmental processes of ophiuroids, including fertilization and early cleavage, are very limited, due to the difficulty in artificially inducing spawning in the laboratory. Indeed, no chemical substances have been reported to induce spawning in the ophiuroids, whereas KCl and 1-methyladenine will induce spawning in other echinoderm groups.

Recently, however, the author has developed a method to induce spawning in the brittle-star *Amphipholis kochii* and has confirmed that this species shows a typical ophiuroid developmental pattern with a free-living ophiopluteus. The present paper describes the embryonic development of the brittle-star *Amphipholis kochii,* from fertilization to metamorphosis, in laboratory culture, with special reference to the early developmental processes.

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MATERIALS AND METHODS

The brittle-star, *Amphipholis kochii,* was found under stones between the tidemarks at Abuta on the Pacific coast of southwestern Hokkaido, Japan. Samples of the mature animals were taken during their breeding season in June and July (Iwata and Yamashita, 1982; Yamashita and Iwata, 1983).

Fertilization of eggs in the laboratory was carried out according to the method previously described (Yamashita, 1983). Embryos and larvae were reared in sea water filtered through a 0.45-μm Millipore filter and treated with antibiotics, penicillin G (100 units/ml), and streptomycin sulfate (1 mg/ml). Artificial sea water (Jamarin U; Jamarin Lab., Osaka, Japan) was also used for the culture medium. The culture medium was gently stirred (*ca.* 60 rpm) as in echinoplutei cultures (Hinegardner, 1969), and was changed daily during early development and every other day during later stages. The temperature of the medium was maintained at 23°C or 15°C. Larvae were fed laboratory cultures of the diatom, *Phaeodactylum tricornutum.*

The embryos and larvae were taken from the culture dish at appropriate intervals and observed under a light microscope. To analyze the cleavage pattern of the early embryos, several fresh samples were also observed by means of a time-lapse cinematograph.

For histological observation, the embryos and larvae were fixed in Bouin's solution for three hours or more, dehydrated with ethanol, and embedded in paraffin. The serial sections (3–5 μm) were stained with Delafield's hematoxylin and eosin.

RESULTS

Gamete shedding and early development

The brittle-star *Amphipholis kochii* is induced to shed gametes by the temperature shock described previously (Yamashita, 1983). Movement of the brittle-star becomes active after the temperature shock, and 30–40 min following the temperature elevation from 4°C to 23°C, the brittle-star sheds all gametes through five-paired bursal slits. Each female (1 mm disk diameter and containing 70–80 ovaries) spawns about 40,000 eggs. When both males and females are kept in a finger bowl and treated with the temperature shock, males usually spawn before the females. This suggests that the shedding of sperm prior to that of eggs may take place in the field. The shedding posture is similar to those found in other ophiuroids; the disk is raised several centimeters above the bottom and contracts vigorously (Fig. 1) (Mortensen, 1920; Olsen, 1942; Woodley, 1975; Hendler, 1977; Mladenov, 1979; Bowmer, 1982; Hendler and Meyer, 1982).

The spawned eggs are at the first meiotic metaphase and remain in that stage until fertilization (Fig. 2A). The living unfertilized egg, about 90 μm in diameter, is opaque, brownish red, and homolecithal (Fig. 2A). It is slightly heavier than sea water. A transparent jelly coat, measuring 5 μm thick, entirely surrounds the egg surface (Fig. 2A). The unfertilized eggs are capable of forming a fertilization membrane for at least 3 hours after spawning, but the ability of the eggs to develop normally diminishes as fertilization is delayed (Fig. 3).

Fertilization occurs at any point on the egg surface. The spermatozoa which fail to contribute to fertilization remain attached to the surface of the jelly coat (Fig. 4). As soon as cortical reaction occurs, the jelly coat overlying the sperm entry site begins to dissolve. It completely disappears from the whole egg surface within a few minutes postinsemination (Fig. 4). When the jelly coat of the unfertilized eggs is removed by acidic sea water (pH = 5.0), these eggs are no longer fertilizable even in normal sea

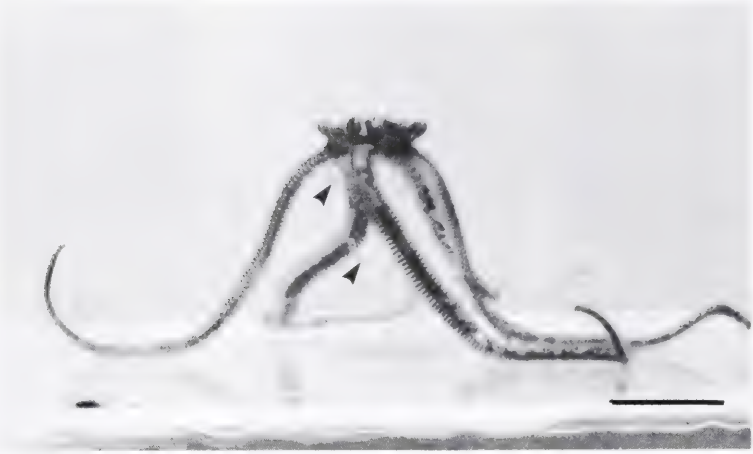

FIGURE 1. Shedding posture of *Amphipholis kochii* female induced to spawn by temperature shock. Arrowheads indicate spawned eggs. Shedding posture of the male is the same. Scale, 1 cm.

water, suggesting that the jelly coat plays an important role in fertilization. However, that the eggs were irreparably damaged by acidic sea water cannot be ruled out.

The fertilized egg has a transparent fertilization membrane and a translucent, thick (7–8 μm) hyaline layer (Fig. 2B). The fertilization membrane is not sticky. The first and second polar bodies, measuring 3–4 μm in diameter move freely within the perivitelline space (Fig. 2C). Occasionally the first polar body subdivides into two, so that three polar bodies are seen in the perivitelline space.

Changes in the egg morphology before, during, and after fertilization as observed with transmission and scanning electron microscopes have been described elsewhere (Yamashita, 1983, 1984).

Culture of the embryos and larvae was carried out at 23°C and 15°C, since the field temperature during the breeding season of the present species ranges from 15°C to 23°C (*cf.*, Yamashita and Iwata, 1983). The following description of the embryonic development is based on the culture at 23°C. There is no significant difference between the two cultures, except for developmental speed. The rate of development in the two cultures, together with the Q_{10}, is shown in Table I. The failure to achieve metamorphosis at 15°C (Table I) is not an effect of temperature. It is probably due to unsuitable conditions, caused by long-term culture.

Cleavage is holoblastic and equal. Unequal cleavage, as observed in echinoid development, is not found in this species. The first division cuts the egg along the longitudinal axis (Fig. 2D). During and after the second cleavage, arrangement of the blastomeres becomes irregular: in many embryos, the blastomeres lie across each other, somewhat resembling the arrangement of the spiral cleavage (Figs. 2F, H). Some embryos show the regular radial arrangement of the blastomeres at the early cleavage stages (Figs. 2E, G), but in all cases the blastomeres become irregularly arranged during cleavage.

Cinematographic analysis reveals that this irregular arrangement is due to intrinsically irregular cleavage planes of each blastomere (Fig. 5), although it also reveals that in some embryos the blastomeres are rearranged after the regular radial cleavage. In any case, the embryos develop quite normally regardless of blastomere arrangement. Because of irregular cleavage and movable polar bodies, it is very difficult to follow

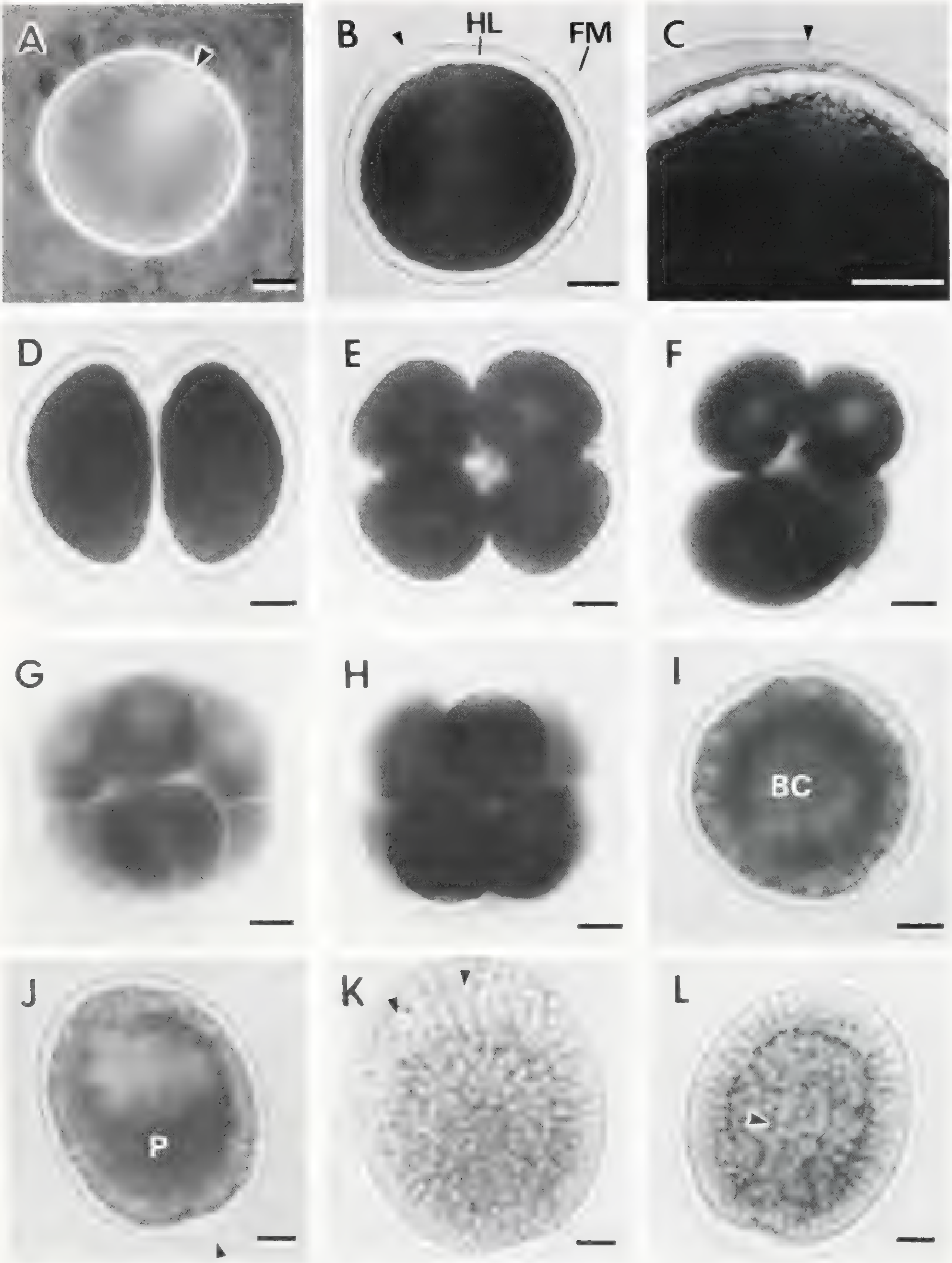

FIGURE 2. Unfertilized egg and early embryos. Scale, 20 μm. A. Unfertilized egg in the sea water containing India ink to show the jelly coat. Arrowhead indicates the region where spindle of the first meiotic metaphase is situated. Lateral view. B. Fertilized egg surrounded by the fertilization membrane (FM) and hyaline layer (HL). Swelling of the hyaline layer at the animal pole (arrowhead) is the elimination site of the first polar body. Lateral view. C. High magnification of the animal polar region of the fertilized egg, showing the first and second polar bodies (arrowhead). D. Two-cell stage embryo. Lateral view. E. Regular four-cell stage embryo. Animal polar view. F. Irregular four-cell stage embryo. Animal polar view. Note the irregular arrangement of the blastomeres. G. Regular eight-cell stage embryo. Lateral view. H. Irregular eight-cell stage embryo. Lateral view. I. Blastula having a narrow blastocoel (BC). J. Swimming blastula. Primary mesenchyme (P) migrates to the blastocoel. The hyaline layer is thickest at the posterior pole (arrowhead). K. Swimming blastula slightly compressed to show vacuoles in the animal polar wall (arrowheads). Primary mesenchyme has occupied almost the whole cavity of the blastocoel. L. Gastrula. Arrowhead indicates the archenteron.

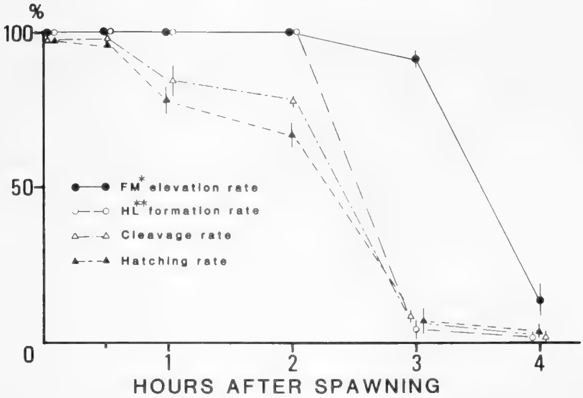

FIGURE 3. Relationship between the ability of the egg to develop normally and the time of insemination after the spawning at 23°C. Measurement was made three times and about 100 eggs were examined in each measurement. Mean ± standard deviation. FM*, Fertilization membrane; HL**, Hyaline layer.

cell lineage. Successive divisions occur at approximately 40-min intervals, producing a spherical blastula with a narrow blastocoel (Fig. 2I).

The embryos hatch in the blastula stage. Usually the swimming blastulae gather near the water surface. A newly hatched, swimming blastula is still surrounded by the

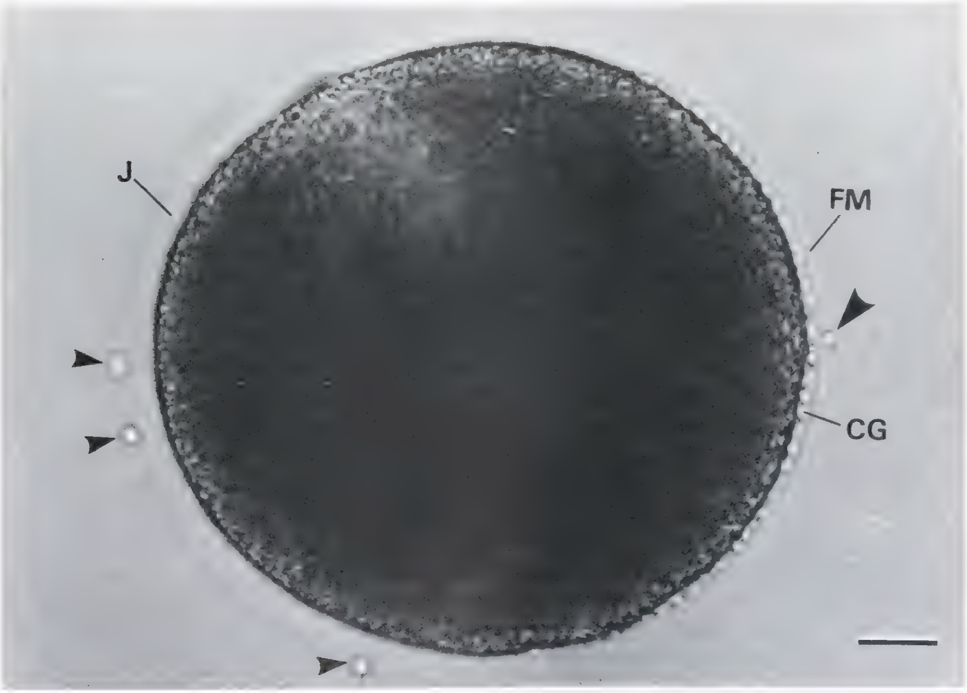

FIGURE 4. An egg during fertilization. The spermatozoon entering the egg is indicated by a large arrowhead. The other spermatozoa (small arrowheads) are attached to the jelly surface and are unable to enter the jelly coat (J). The discharged cortical granules (CG) and developing fertilization membrane (FM) are also seen. Note that the jelly coat begins to dissolve at the region of sperm entry. Scale, 10 μm.

TABLE I

Timetable for the embryonic development of Amphipholis kochii *egg cultured at 23°C and 15°C*

Stage	23°C	15°C	Q_{10}
First polar body	15 (min)	—	—
Second polar body	30	—	—
Two-cell	1.5 (h)	2 (h)	1.43
Four-cell	$2\frac{1}{6}$	3	1.50
Eight-cell	$2\frac{5}{6}$	4	1.54
Blastula	7–8	10–12	1.32–1.96
Hatching	8.5	13–14	1.70–1.87
Gastrula	13–17	1 (day)	1.53–2.15
Spicule formation	21	1.5	1.96
Archenteron differentiation	1.1 (day)	1.8	1.85
Coelomic pouch formation	1.3	2	1.71
Two-armed ophiopluteus	1.7	2	1.22
Left posterior coelomic pouch formation	1.7	3	2.03
Four-armed ophiopluteus	2	4	2.38
Right posterior coelomic pouch formation	2.5	6.5	2.98
Six-armed ophiopluteus	4.5	8.5	2.21
Eight-armed ophiopluteus	6	—	—
Hydrocoel formation	6.5	10.5	1.82
Hydrocoel five-lobed	8.5	—	—
Metamorphosis (incipient)	12	—	—

thick hyaline layer which is thickest at the posterior pole (Fig. 2J). Cells in the vegetal polar wall migrate into the blastocoel and occupy almost the whole cavity (Figs. 2J, K; 7A). These are probably the primary mesenchymal cells. Concurrently with the

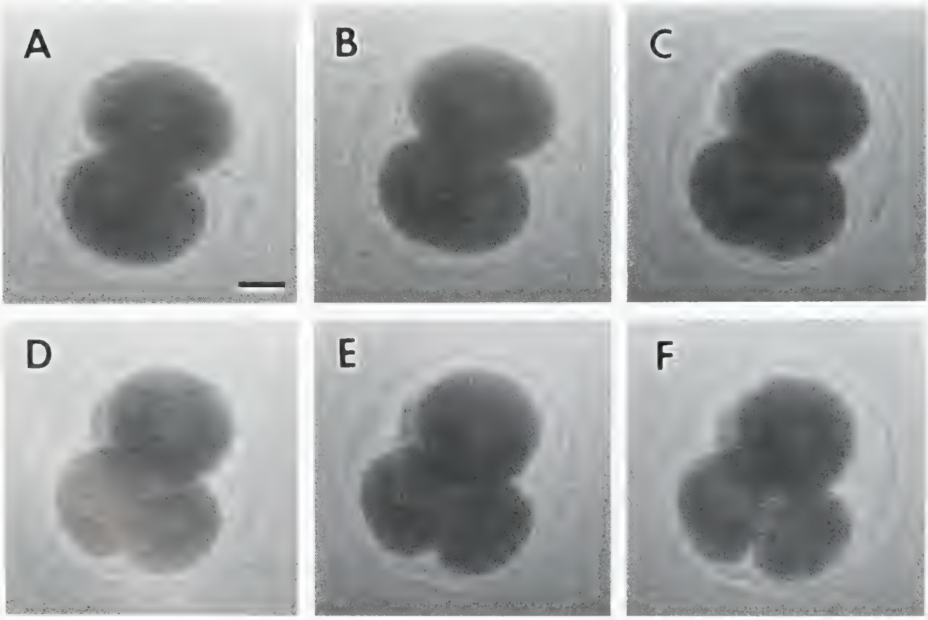

FIGURE 5. The second cleavage as observed by time-lapse cinematography. The second cleavage planes in two blastomeres are perpendicular to each other, producing an irregular arrangement of the blastomeres. Photographs were taken from 1 min intervals in the film. Scale, 20 μm.

cellular migration, the spherical swimming blastula becomes dorso-ventrally flattened and ellipsoidal in shape (Figs. 2J, K). The many vacuoles in the animal polar wall (Figs. 2K, 7A) may serve as a floating device, as suggested by Narasimhamurti (1933).

Gastrulation was achieved by an invagination of the wall cells at the vegetal pole (Figs. 2L, 7B, C). During gastrulation, the embryo becomes more dorso-ventrally flattened, taking a shield-like shape (Fig. 2L). At this stage, spicules begin to form in the primary mesenchyme (Fig. 6A), and take a tetraradiate shape (Fig. 6B).

Later development and metamorphosis

The early two-armed ophiopluteus is helmet shaped due to the appearance of the postero-lateral arms (Fig. 6C). Brownish red pigments are first observed in this larva, especially at the tip of the larval arms. At this stage, the archenteron differentiates into an intestine, stomach, and esophagus (Fig. 6C). The right and left coelomic pouches, which are derived from two pockets of the archenteron (Fig. 7C), are found near the constricted region between the stomach and esophagus (Fig. 6C). The antero-lateral arms are the second pair formed (Fig. 6D). The left posterior coelom is detected in the early four-armed ophiopluteus (Fig. 6D). It is uncertain whether the posterior coelom is formed by segregation of the original coelomic pouch. The stomach enlarges during the four-armed stage, after which the larva begins to eat diatoms. At the age of 4.5 days, the larva produces the post oral arms, resulting in a six-armed ophiopluteus (Fig. 6E). Finally, in the 6-day-old larva, the fourth arms, the postero-dorsal arms, are formed (Fig. 6G).

The eight-armed ophiopluteus reaches 350 μm in length along the antero-posterior axis, excluding the length of the antero-lateral arms. The larval skeletal system of *A. kochii* is very simple, without having any of the accessory rods described in other ophioplutei (Fig. 8; *cf.*, Mortensen, 1921). In the eight-armed larva, the left posterior coelom divides into a hydrocoel and a somatocoel (Fig. 7D). The left anterior coelom develops as an axocoel. The hydrocoel expands anteriorly along the stomach and differentiates into five lobes at the side of the esophagus (Figs. 6H, 7E). Formation of the right posterior coelom occurs in a later stage than that of the left posterior coelom (Table I; Figs. 6F, 7D). Further development of these organs is uncertain in this study.

Metamorphosis of the ophiopluteus is gradual. During the metamorphosis of *A. kochii*, the spicule of the adult skeletal system forms a triradiate shape which continues to branch, finally forming a network of spicules (Fig. 8). The anterior part of the larva shrinks and the larval arms degenerate except for the postero-lateral ones (Fig. 6I). The five-lobed hydrocoel surrounds the esophagus before forming the pentaradial water canal system of the adult brittle-star. As a result of these modifications, the anterior part of the larva develops into a young brittle-star which bears several podia (Fig. 6I). At the completion of metamorphosis, the last larval arms, the postero-lateral arms, degenerate.

DISCUSSION

In the ovary of *A. kochii*, we were unable to find secondary oocytes or ova even during the breeding season (Iwata and Yamashita, 1982). Since the spawned eggs remain in the metaphase of the first meiotic division, oocyte maturation, including the breakdown of the germinal vesicle and the entrance into the meiotic division, occurs immediately before spawning. In fact, the ovarian oocytes show the germinal vesicle breakdown after the temperature shock (unpub. obs.). A similar situation for

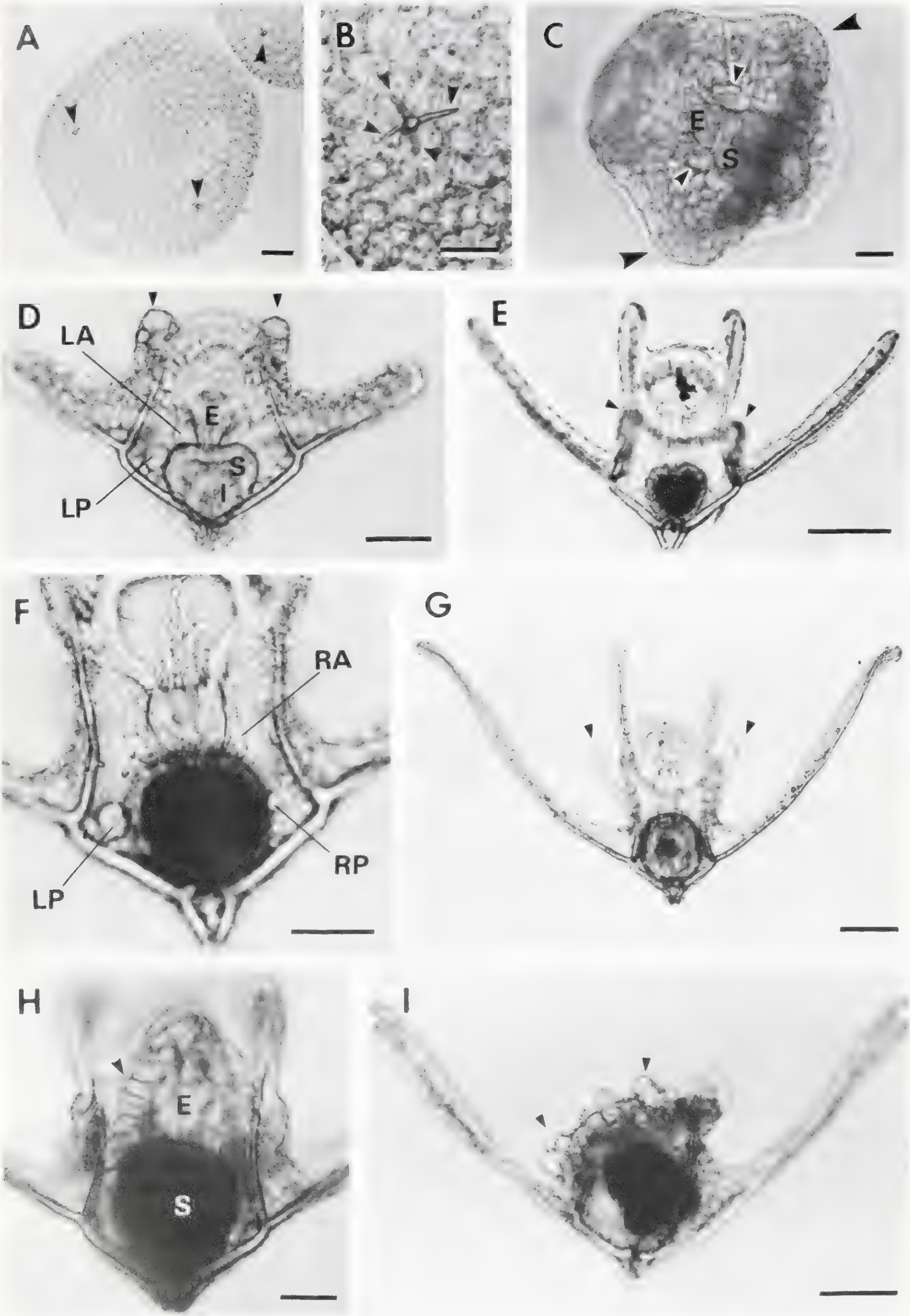

FIGURE 6. Gastrula and ophioplutei from the early two-armed to metamorphosis stage. Scale, 10 μm (B), 20 μm (A, C), 50 μm (D, F, H), 100 μm (E, G, I). A. Gastrula compressed to show the tetraradiate spicules (arrowheads). B. High magnification of the tetraradiate spicule showing the rudiments of the four larval skeletal rods (arrowheads). C. Early two-armed ophiopluteus. Large arrowheads indicate the rudiments of the postero-lateral arms. Apparent coelomic pouches (small arrowheads) are found between the stomach (S) and esophagus (E). The intestine is out of focus. Ventral view. D. Early four-armed ophiopluteus. The

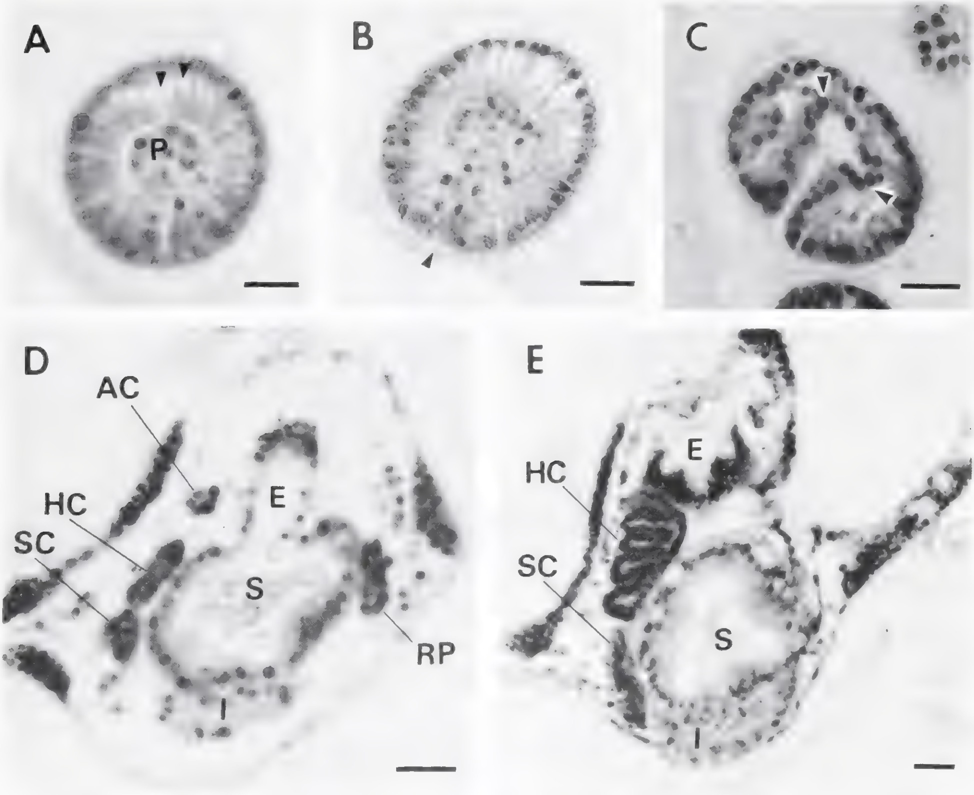

FIGURE 7. Histological longitudinal sections of the embryos and larvae. Scale, 20 μm. A. Blastula. Primary mesenchyme (P) has occupied the blastocoel. Arrowheads show the vacuoles in the animal polar wall. B. Early gastrula, showing invagination at the vegetal pole (arrowhead). C. Late gastrula. Archenteron has formed. Arrowheads point out two pockets in the archenteron, which are the origin of the coelomic pouches. D. Eight-armed ophiopluteus. The left posterior coelom has divided into hydrocoel (HC) and somatocoel (SC). AC, axocoel; E, esophagus; I, intestine, RP, right posterior coelom; S, stomach. E. Late eight-armed ophiopluteus. The hydrocoel (HC) has developed along the esophagus (E) and become a five-lobed shape. The somatocoel (SG) has grown at the side of the stomach (S). I, intestine.

oocyte maturation has been reported in the asteroids, *Asterias amurensis* and *Asterina pectinifera*, in which oocyte maturation is induced by the neurosecretory system immediately before spawning (*cf.*, Kanatani, 1973).

It is notable that the spawning of the asteroids, induced by a gonad stimulating substance (GSS) from the radial nerve, also has a time lag of similar duration to the period observed after the temperature shock of *A. kochii* (Kanatani and Ohguri, 1966). This similarity implies that the temperture shock given for the spawning of *A. kochii*

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antero-lateral arms are indicated by arrowheads. The left anterior (LA) and posterior (LP) coeloms are seen. The larval digestive system has developed into the esophagus (E), stomach (S), and intestine (I). Dorsal view. E. Six-armed ophiopluteus. The third arms, post oral arms (arrowheads), are seen. Ventral view. F. Six-armed ophiopluteus, in which the right anterior (RA) and posterior (RP) coeloms are seen. The left posterior coelom is also seen (LP). Dorsal view. G. Eight-armed ophiopluteus. The postero-dorsal arms (arrowheads) are prominent. Dorsal view. H. Ophiopluteus with five-lobed hydrocoel (arrowhead). The esophagus (E) and stomach (S) are also indicated. I. Ophiopluteus during metamorphosis. Arrowheads indicate the podia of a young brittle-star. Dorsal view.

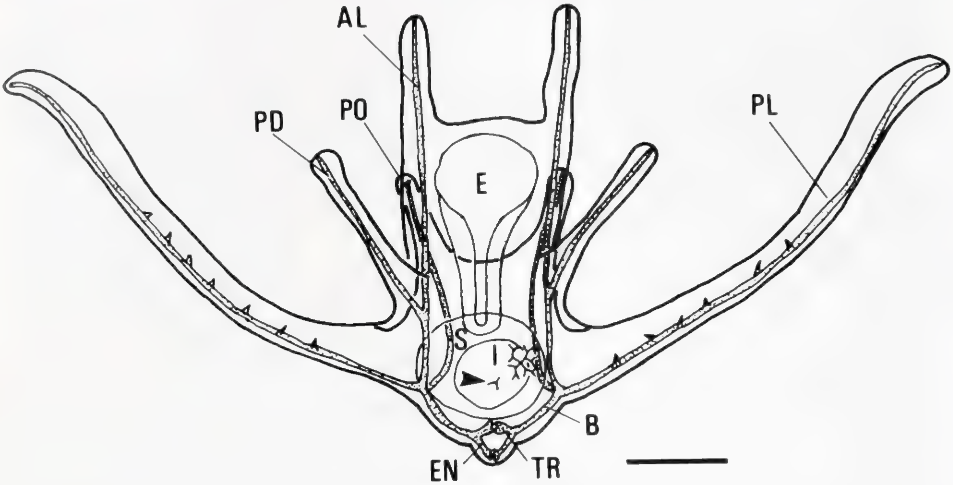

FIGURE 8. Eight-armed ophiopluteus, showing a larval skeletal system. For simplicity, coeloms and other small structures were omitted. Dorsal view. Arrowheads indicate the spicules of adult skeletal system. AL, antero-lateral rod; B, body rod; E, esophagus; EN, end rod; I, intestine; PD, postero-dorsal rod; PL, postero-lateral rod; PO, post oral rod; S, stomach; TR, transverse rod. Scale, 100 μm.

directly induces the nerve to release the GSS. However, it is still uncertain whether oocyte maturation and the spawning of the ophiuroids are controlled by the neurosecretory system as in the asteroids, although Fontaine (1962) has shown the presence of the neurosecretory cells in the radial nerve of the ophiuroid. Therefore, further experimental studies, such as *in vitro* maturation of ophiuroid oocytes, are necessary to clarify the mechanism controlling oocyte maturation and the spawning of ophiuroids.

The irregular arrangement of the blastomeres observed in the early cleavage stages of *A. kochii* egg has also been described in such ophiuroids as *Ophiopholis aculeata, Ophiothrix fragilis,* and *Ophiura albida.* It has been suggested that the irregularity of the blastomere arrangement is induced by the pressure exerted by the hyaline layer (Olsen, 1942). The fertilization membrane- and hyaline layer-free embryos (denuded embryos) of *A. kochii* obtained by treating with Ca-Mg-free sea water shows a mass of the blastomeres, which is easily scattered (unpub. obs.), as is the case of the denuded embryo of the asteroid (Dan-Sohkawa, 1976). It is therefore apparent that the hyaline layer plays an important role in the arrangement of the blastomeres in the ophiuroid embryo. However, the present study also demonstrates that the irregular arrangement of the blastomeres is caused not only by rearrangement of the blastomeres after regular radial cleavage, which is probably induced by the thick hyaline layer, but by an intrinsic, irregular cleavage plane in the blastomeres. An intrinsic irregular cleavage in the early embryos has been also found in many mammals (Gulyas, 1975). Moreover, various kinds of cleavage patterns, *e.g.,* irregular arrangement of blastomeres as observed in this study, or fusion of blastomeres after cleavage, have been reported in the asteroid embryos which develop quite normally (Teshirogi and Ishida, 1978). The irregular arrangement of blastomeres during the early cleavage may be an unusual pattern in animal eggs, but further detailed observations on the early cleavage of additional species are necessary.

As a rule, the larval spicules of the ophioplutei take a triradiate form (*cf.,* Mortensen, 1921). Therefore, the tetraradiate spicule present in *A. kochii* is remarkable when compared with most other reported ophioplutei. The triradiate spicule in other ophioplutei consists of the antero-lateral, postero-lateral, and body rods, and the post oral rods are formed in the later stage of embryonic development. In *A. kochii*, however, the post oral rod is formed at the same stage as that of the earlier three rods. This precocious formation of the post oral rod gives the spicule of *A. kochii* a tetraradiate shape and may be related to a relatively rapid development of *A. kochii* as compared with other typical ophioplutei (*cf.*, Hendler, 1975). The simple skeletal system of this larva also seems to reflect the relatively rapid embryonic development of this species.

Other *Amphipholis* species hitherto studies are all littoral and viviparous (Mortensen, 1920; Fell, 1946; Oguro *et al.*, 1982; see also Hendler, 1975). The present study, however, reveals a littoral *Amphipholis* species with rapid, planktotrophic development. This confirms the notion that the developmental pattern in ophiuroids is related to ecological rather than phylogenetic factors (Fell, 1966; Nichols, 1969; Hendler, 1975).

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GENETIC VARIATION IN POPULATIONS OF THE HERMAPHRODITIC FLATWORM *MESOSTOMA LINGUA* (TURBELLARIA, RHABDOCOELA)

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ABSTRACT

Populations of the rhabdocoel *Mesostoma lingua* at a site in the Canadian arctic were polymorphic at two of ten allozyme loci. Phenotypic frequencies were determined in more than 1500 individuals from 35 populations at the polymorphic loci phosphoglucomutase and mannose-6-phosphate isomerase. Considerable gene frequency divergence was noted between populations only a few meters apart indicating that gene flow is low. Inbreeding coefficients due to population subdivision averaged 0.15, suggesting that existing populations receive an average of only one new migrant per generation. Genotypic frequencies in individual populations were ordinarily in good agreement with Hardy-Weinberg expectations, revealing that this hermaphroditic organism does not engage in self-fertilization.

INTRODUCTION

Simultaneous hermaphroditism is common in animal groups whose adult dispersal is limited either by a sessile life-style or by the occupation of a patchy habitat such as a pond or a host organism. Ghiselin (1969) has pointed out the obvious adaptive significance of self-fertilization in such organisms, particularly in habitat colonization. Laboratory studies have shown that many simultaneous hermaphrodites are capable of self-fertilization (Gee and Williams, 1965; Brenner, 1974). The few studies on natural populations of such hermaphrodites suggest that the incidence of self-fertilization can be high. Selander and Hudson (1976) showed that the gastropod *Rumina decollata* is ordinarily self-fertilizing and that natural populations consist of a number of genetically distinct, inbred lines comparable to those which exist in self-compatible plants (Jain, 1976). Similar observations have been made on a variety of hermaphroditic organisms including fish (Harrington and Kallman, 1968), anemones (Cain, 1974), and polychaetes (Beckitt, 1982).

The breeding systems of some major hermaphroditic groups, such as the turbellarian flatworms have been little studied. This class includes five orders of which the triclads and rhabdocoels are best known. Most triclads are obligate outcrossers, as isolated individuals fail to produce viable eggs (Hyman, 1951). However, members of the genus *Procerodes* are capable of self-fertilization and the freshwater triclad *Cura foremannii* is an obligate selfer (Anderson and Johann, 1958). Members of the rhabdocoel family Typhloplanidae typically produce two sorts of eggs—subitaneous and diapausing. The subitaneous eggs develop immediately and are thought to be self-fertilized, while the diapausing eggs are ordinarily thought to be cross-fertilized (Hyman, 1951). It is known, however, that some rhabdocoels are capable of producing resting eggs by self-fertilization (Sekera, 1906).

Although a large amount of work has been done on the cytogenetics of turbellarians (Bennazzi and Lentati, 1976) few studies of genetic variation in natural populations

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have been attempted. Biersma and Wijsman's (1981) survey of several European populations of the congeneric triclads *Polycelis nigra* and *P. tenuis* revealed variation at two enzyme loci. The patterns of variation did not, however, permit a simple genetic interpretation of their results. Other studies have examined the extent of genetic divergence among triclad taxa (Nixon and Taylor, 1976), but no studies of population structure have been attempted. The present study aimed to examine both the extent of inbreeding within populations and the amount of genetic divergence among local populations of the rhabdocoel flatworm, *Mesostoma lingua* (Abildgaard 1789). The genus *Mesostoma* includes more than 50 species (Ferguson and Hayes, 1941), and has a global distribution. Members of the genus typically produce both resting and subitaneous eggs. The subitaneous eggs are fragile, while the resting eggs are enclosed within a strong membrane. The former eggs initiate development immediately, while resting eggs are responsible for survival during periods of unfavorable climatic conditions. Members of the genus differ from most other turbellarians in being active predators on zooplankton as well as benthic invertebrates (Maly *et al.*, 1980; Schwartz and Hebert, 1982; MacIsaac and Hutchinson, 1985). *M. lingua* is broadly distributed through Eurasia (Ferguson and Hayes, 1941) and in the subarctic portions of North America (Holmquist, 1976). In Europe the species produces a series of generations via subitaneous egg formation (Heitkamp, 1977), but populations in northern Canada are univoltine (pers. obs.). Juveniles emerge from diapausing eggs in early June and are mature by mid–late July. Individuals produce a brood of resting eggs within the body and release them when they die in late August. The present study involved a survey of enzyme variability at two polymorphic loci in populations of *M. lingua* in pond habitats near Churchill, Manitoba.

MATERIALS AND METHODS

Ponds were surveyed for *M. lingua* on two quartzite rock bluffs (A and C) located approximately 22 km east of Churchill, Manitoba. These bluff ponds have been the subject of detailed water chemistry and zooplankton distribution studies (Hebert and Billington, in prep.). These two bluffs are approximately 1.7 km apart, and have surface areas of 26,036 and 141,344 m^2 respectively. Some of the bluff ponds appear to sit within glacial scours, while others lie in fractures in the rock surface. The ponds on both bluffs had a similar mean surface area (≈ 15 m^2) and a similar average depth (≈ 0.4 m). The ponds show considerable variation in salinity, as a result of their varying proximity to Hudson Bay. The salinity variation reflects inputs of sea-spray as opposed to direct tidal flushing of the ponds. Many of the ponds are intermittent in years of low rainfall. Sixty ponds were present on Bluff A and 184 ponds on Bluff C.

The presence or absence of *Mesostoma lingua* in each pond was assessed by trapping during the period June 15–July 23, 1984. The traps were 100 ml jars whose lids were modified by the insertion of a 4 cm diameter piece of 1000 μm nylon mesh. Approximately 100 heat-killed zooplankton (*Diaptomus, Daphnia*) were placed inside each jar as bait. After baiting, the jars were placed on their side in the pond and marked with a float for easy recovery. Two traps were initially placed in each pond and left for a 24-hour period. If *Mesostoma* were not present when these traps were removed, the procedure was repeated on at least two occasions. In ponds containing *Mesostoma*, a single jar often trapped more than 100 animals, and both traps ordinarily contained animals.

Electrophoresis was carried out using cellulose acetate gels. Gels were presoaked for 30 minutes in pH 8.5 Tris Glycine (3 g Trizma, 14.4 gm Glycine in 1 l H_2O).

Mesostoma were macerated individually in sample wells and 12–24 samples applied to each gel plate. Gels were run for 15 minutes at 200 volts using a Tris Glycine buffer of the same molarity and pH as that used for soaking. Gels were stained using an agar overlay and standard staining solutions.

Five populations were screened for variation at ten loci including phosphoglucose isomerase, phosphoglucomutase (PGM), mannose phosphate isomerase (MPI), aldehyde dehydrogenase, lactate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate oxaloacetate transaminase, amylase, and triose phosphate isomerase. Polymorphism was detected at only two loci—PGM and MPI. As MPI migrated more anodally than PGM it was possible to stain both enzymes on a single gel plate by using a stain mixture that contained the substrates and coupling agents for both enzymes. When possible, a sample of 48 individuals was electrophoresed for MPI and PGM from each of the ponds containing *Mesostoma*. These samples were collected between July 3 and 23, 1984, and included both late juvenile and adult individuals.

Electrophoretic analysis showed that recently fed *Mesostoma* contained active enzymes from their prey (Hebert *et al.*, in prep.). These enzymes were degraded within 2 days at 20°C, so collections of *Mesostoma* were generally held for several days at room temperature before electrophoresis.

Inbreeding coefficients were calculated using standard methods (Wright, 1978). $F_i = 1 - H_o/H_e$ where H_o is the observed heterozygosity and H_e is the amount expected at Hardy Weinberg equilibrium (H.W.E.). F_{is} was the weighted mean of the F_i values (*i.e.*, $F_{is} = \sum_{1}^{N} (n_i/n_t) F_i$ where n_i is the number of individuals sampled in a population, n_t is the total sample size; and N is the number of populations surveyed). The degree of divergence among populations was quantified using a hierarchical F analysis as described by Wright (1978). Three levels were recognized in the hierarchical analysis (demes, subdivisions, and total). The population of *Mesostoma* in an individual pond was treated as a deme, while the pooled populations on each of the two rock bluffs were treated as subdivisions of the total Churchill population. Based on gene frequency data, variances were calculated for gene frequencies among the populations in each subdivision (s^2_{DS}), and between subdivisions in the total (s^2_{ST}) and among demes in the total population (s^2_{DT}). Variances were corrected for sampling error as described in Wright (1978, pp. 86–89). The sampling variance for demes was, for instance, calculated using the formula $SE^2 = \frac{1}{N} \sum_{1}^{N} [q(1 - q)/n]$, where n is the number of genes sampled from a population, q is the frequency of the slow allele in a population, and N is the number of populations sampled. From the corrected variance estimates, inbreeding coefficients were calculated using the following formulae: $F_{DT} = s^2_{DT}/\bar{q}(1 - \bar{q})$; $F_{ST} = S^2_{ST}/\bar{q}(1 - \bar{q})$; and $F_{DS} = F_{DT} - F_{ST}/(1 - F_{ST})$ where $\bar{q}$ is the mean frequency of the slow allele in all populations. In the interpretation of these inbreeding coefficients it has been assumed that the allozyme variants at PGM and MPI have little or no effect on individual fitness.

RESULTS

Distribution

M. lingua was the only large-bodied (>5 mm) rhabdocoel collected in the traps. Thirteen of the 60 ponds on Bluff A and 24 of the 184 ponds on Bluff C contained

M. lingua. Densities of the species in two (1, 38A) of the Bluff A ponds and in three (20, 29, 156) of the Bluff C ponds were too low to permit collection of the desired sample size for electrophoresis.

Allozyme variation

Twenty-four individuals from each of 3 ponds on Bluff A (4, 20, 60) and 2 ponds on Bluff C (13, 72) were examined for allozyme variation at 10 loci. Each population was monomorphic for the same allele at eight loci, but all were polymorphic at PGM and most were polymorphic at MPI. Phenotypic variation at both these loci could be explained by assuming the presence of two alleles. Homozygous phenotypes were single-banded and heterozygous phenotypes double banded, as expected on the basis of the quaternary structure of these enzymes.

Gene frequency variation among populations

Allozyme phenotypes at PGM and MPI were determined for more than 500 individuals from Bluff A and for more than 1000 individuals from Bluff C (Tables I, II). Twenty-four or more individuals were analyzed for each enzyme in 32 of the 35 populations investigated. Pronounced gene frequency variation was noted among these populations. Frequency of the slow allele of PGM varied among Bluff A populations from 0.25 to 0.76, and among Bluff C populations from 0.08 to 0.93. The variation in gene frequencies among Bluff A populations was highly significant ($G_{adj} = 80.57$, d.f. = 20, $P < 0.001$), as was that among Bluff C populations ($G_{adj} = 350.7$, d.f. = 40, $P < 0.001$). The mean frequency of the S allele of PGM on Bluff A did not differ from that on Bluff C ($G_{adj} = 0.94$, $P > 0.30$). Similar gene frequency variation was noted at MPI with the frequency of the slow allele varying from 0.46 to 0.94 on Bluff A and from 0.49 to 1.00 on Bluff C. The mean frequency of the S allele was significantly higher on Bluff C than on Bluff A ($G_{adj} = 77.9$, $P < 0.001$).

TABLE I

Genotypic frequencies at PGM and MPI for populations of M. lingua *on Bluff A*

	PGM Frequencies					MPI Frequencies				
	Genotypes			Gene		Genotypes			Gene	
Pond	SS	SF	FF	S	F_i	SS	SF	FF	S	F_i
A1	0	3	5	–	–	6	3	0	–	–
A3	15	21	12	.53	.12	38	10	0	.90	−.12
A4	10	23	14	.56	.01	38	10	0	.90	−.12
A5	28	17	3	.76	.03	9	26	13	.46	−.09
A6	10	23	15	.45	.03	15	20	13	.52	.17
A9	15	16	5	.64	.04	23	20	5	.69	.03
A20	4	22	22	.31	−.07	12	24	12	.50	0
A21	11	22	14	.47	.06	12	21	14	.48	.10
A38B	5	14	28	.25	.22	27	19	2	.76	−.09
A44	15	20	13	.52	.17	19	20	7	.63	.07
A45	11	25	11	.50	−.06	33	15	0	.89	−.19
A60	10	37	35	.35	−.005	51	7	0	.94	−.06
Total	134	243	177	.46		283	195	66	.70	

TABLE II

Genotypic frequencies at PGM and MPI for populations of M. lingua *on Bluff C*

	PGM Frequencies					MPI Frequencies				
	Genotypes			Gene		Genotypes			Gene	
Pond	SS	SF	FF	S	F_i	SS	SF	FF	S	F_i
C1	4	28	16	0.38	−.24	18	24	6	0.63	−.07
C13	13	37	24	0.43	−.02	73	1	0	0.99	−.001
C20	4	3	2	—		6	3	0	–	
C28	14	25	9	0.55	−.05	10	30	8	0.52	−.25
C29	3	5	7	–		6	4	5	–	
C37	9	29	10	0.49	−.21	12	23	13	0.49	.04
C40	10	27	11	0.49	−.13	39	9	0	0.91	−.10
C41	7	28	13	0.44	−.19	35	11	2	0.84	.13
C59	4	16	25	0.27	.09	31	11	1	0.85	.003
C60	18	24	6	0.63	−.07	39	8	1	0.90	.11
C61	6	25	17	0.39	−.10	37	11	0	0.89	−.13
C65	11	28	9	0.52	−.17	34	13	1	0.84	−.03
C66	38	8	2	0.88	.24	14	25	9	0.55	−.05
C70A	55	9	0	0.93	−.08	62	2	0	0.98	−.02
C72	9	31	12	0.47	−.20	52	0	0	1.00	
C83	0	6	30	0.08	−.09	21	3	0	0.94	−.07
C89	7	27	13	0.44	−.17	30	15	3	0.78	.08
C98	16	24	8	0.58	−.03	47	1	0	0.99	−.01
C100	4	17	18	0.32	−.001	26	6	0	0.99	−.10
C111	2	19	27	0.24	−.09	40	8	0	0.92	−.09
C134	13	21	14	0.49	.12	46	2	0	0.98	−.02
C134A	3	21	28	0.26	−.05	52	0	0	1.00	
C151	13	25	10	0.53	−.05	25	23	0	0.76	−.32
Total	263	483	311	0.48		755	234	49	0.84	

No microgeographical pattern was obvious in the gene frequency variation among populations on Bluffs A and C (Figs. 1, 2). Substantial gene frequency differences were, however, evident between populations only a few meters apart. Hierarchical F-analysis revealed that the pattern of gene frequency divergence was similar at the two loci (Table III). The large proportion of inbreeding was attributable to differences among populations on the same bluff, with at most a small contribution due to differences among bluffs. The mean inbreeding coefficient (F_{ST}) for the two loci was 0.151.

Genotypic frequencies in individual populations

Tests of genotypic frequencies at both the PGM and MPI loci in the 32 populations with large population sizes revealed only a single significant deviation from H.W.E., due to a heterozygote excess at the MPI locus in population C151. If there were a consistency in the direction of deviation from H.W.E., the mean of the F_i values (Tables I and II) would deviate significantly from zero. Analysis indicated however that these means (−0.03, PGM and −0.04 MPI) did not deviate significantly from zero ($t = -1.37$, $P > 0.10$ at PGM; $t = -2.02$, $P > 0.05$ at MPI). The F_{is} values corresponded closely with mean F_i values as sample sizes did not vary markedly among populations ($F_{is} = -.030$ for PGM, $F_{is} = -.036$ for MPI).

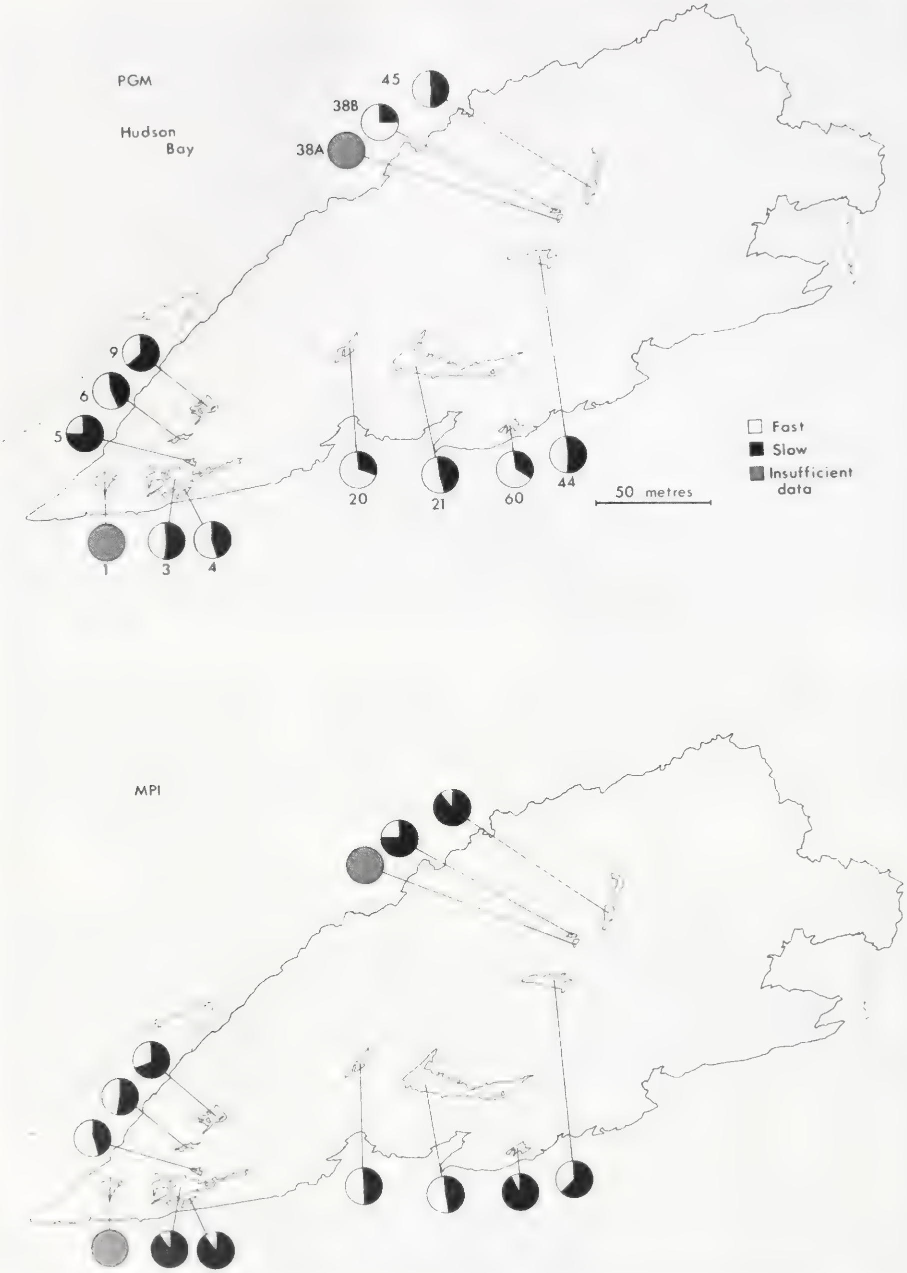
PGM
Hudson Bay
45
38B
38A
9
6
5
1
3
4
20
21
60
44
50 metres
Fast
Slow
Insufficient data
MPI

FIGURE 1. Gene frequency variation at PGM and MPI in populations of *Mesostoma lingua* on Bluff A.

Genotypic frequencies were determined for 9–15 individuals from three (A1, C20, C29) of the five low-density populations. These individuals represented the total population of *Mesostoma* in each habitat, as continued trapping failed to collect additional

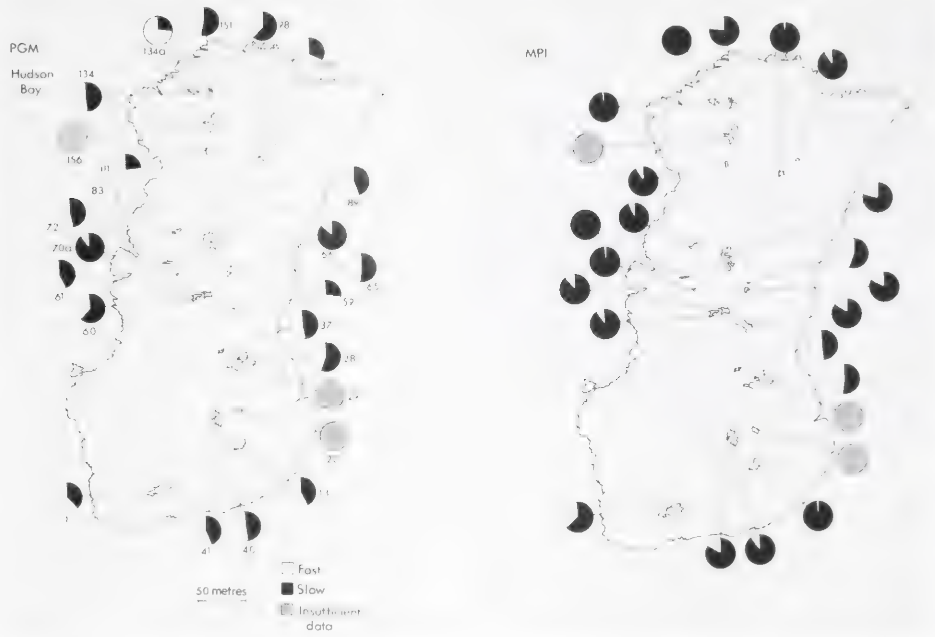

FIGURE 2. Gene frequency variation at PGM and MPI in populations of *Mesostoma lingua* on Bluff C.

specimens. None of these populations showed a significant deviation from H.W.E., although a heterozygote deficiency was evident at both MPI and PGM in the C29 population.

DISCUSSION

Mesostoma lingua is representative of a large number of aquatic invertebrates which rely on an unsophisticated means of dispersal. Not only are its diapausing eggs passively dispersed, but they lack structures to enhance dispersal such as those found in many terrestrial plants and some aquatic organisms (*e.g.*, ephippial cases of cladocerans). There is no direct information on the means by which *Mesostoma* is dispersed. The diapausing eggs remain within the adult's body until it dies, and during this period can be dispersed as a group. Shorebirds are probably an important dispersal

TABLE III

Differentiation of PGM and MPI frequencies among 32 populations of Mesostoma lingua *from 2 rock bluffs.*

	$\bar{q}$	$\Sigma\ SE^2$	$\Sigma\ s^2_{DS}$	$\Sigma\ s^2_{ST}$	$\Sigma\ s^2_{DT}$	$\Sigma\ \bar{q}(1-\bar{q})$	F_{DS}	F_{ST}	F_{DT}
PGM	.47	.0045	.060	−.004	.0561	.498	.120	−.008	.113
MPI	.79	.0028	.057	.006	.062	.330	.175	.017	.189

$\bar{q}$ is the mean frequency of the slow allele, SE^2 is the sampling variance for demes, s^2 is the variance in gene frequencies and F is the inbreeding coefficient. D—deme, S—subdivision, T—Total. Summation signs indicate parameters produced by repeating and summing calculations for each allele (*i.e.*, for both the F and S allele in this case).

agent in intermittent ponds. As such ponds dry, the *Mesostoma* scavenge on the remains of large plankton (*e.g., Branchinecta paludosa*) and are likely to be ingested by birds which select the same food. In permanent ponds dispersal seems unlikely, except via overflow.

Like many other passively dispersed organisms, *Mesostoma lingua* has a broad range. Few studies have examined the amount of population differentiation in such organisms. Although it was long accepted (Mayr, 1963) that gene flow was extensive and local population differentiation minimal in such passively dispersed organisms, allozyme studies on populations of the cladocerans *Daphnia magna* (Hebert, 1974) and *Daphnia carinata* (Hebert and Moran, 1980) indicated marked gene frequency divergence among populations only a few kilometers apart. The generality of these results was questionable, as the parthenogenetic mode of reproduction of *Daphnia* permits populations to be founded from a single individual. However, the extent of local differentiation seen in *M. lingua* rivals that in the cladocerans and suggests that gene frequency divergence may be common among local populations of passively dispersed taxa.

Estimated population sizes of *M. lingua* in several Churchill ponds ranged from 1200–5000 individuals (Hebert, in prep.) indicating that drift is unlikely to be important in explaining the gene frequency differences among populations. Instead, it seems likely that most of the divergence is the result of populations being founded from few individuals. The population structure of *M. lingua* on individual rock bluffs conforms closely to Wright's (1978) island model. Populations are discrete and the probability of gene exchange between each may be similar because of the short distances involved. The mean F_{DS} of 0.15 suggests that populations receive only about 1.4 migrants per generation $\left(F_{DS} = \frac{1}{1 + 4\,N_e m}\right)$. As effective population sizes exceed 1000, migrants appear ordinarily to make up less than 0.1% of a population.

The observation that isolated individuals of *M. lingua* released diapausing eggs suggests that self-fertilization is possible (Hebert, in prep.). However, as attempts to hatch them failed, it was impossible to show that these eggs were viable. The close approach of genotypic frequencies in *M. lingua* populations to H.W.E. indicated that the eggs are rarely if ever self-fertilized in nature. Even if cross-fertilized, some inbreeding would be expected if population sizes were small. The genotypic data provide general evidence that most *M. lingua* populations are large enough so that this does not occur and agrees with the evidence from surveys of population size. It is premature to conclude that the breeding system of *M. lingua* is representative of that of turbellarians or even other rhabdocoels. Studies on the molluscan families Arionidae and Limacidae have shown considerable variation in reproductive behavior among taxa. Some species are facultative selfers, while others are either obligate selfers or outcrossers (McCracken and Selander, 1980; Foltz *et al.*, 1984). Closely related taxa tend to employ a similar breeding system, suggesting that a broad survey of species belonging to different turbellarian families would be the best way of assessing the extent of variation in breeding systems among these organisms.

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IRON ACCUMULATION IN TUNICATE BLOOD CELLS. II. WHOLE BODY AND BLOOD CELL IRON UPTAKE BY *STYELA CLAVA*

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Dedicated to the memory of
Guy C. McLeod

ABSTRACT

Iron accumulation by the tunicate *Styela clava* was investigated. Live specimens did not show uptake of dissolved iron from sea water, although their tunics showed a high affinity for iron adsorption. In partitioning experiments, the tunic of *S. clava* was shown to be impermeable to dissolved iron. The most likely source of iron is particulate matter that tunicates filter through their siphons from sea water.

Iron uptake by *S. clava* blood cells was studied. Iron(II) uptake by the cells was shown to be biphasic. Uptake was shown to be an irreversible process for the time span studied. The metabolic inhibitor 2-deoxyglucose did not inhibit uptake. Citrate blocked uptake. Addition of Ga(III) did not affect uptake, implying that iron(II) does not undergo oxidation changes during accumulation by the blood cells. Uptake did not occur through anionic channels. Iron(III) uptake studies were inconclusive because of the low solubility of uncomplexed iron(III) species.

INTRODUCTION

Most ascidians are known for the high content of vanadium in their blood and for their ability to concentrate vanadium from sea water. However some ascidians lack vanadium, but contain high concentrations of iron in their blood (Endean, 1953; Agudelo *et al.*, 1983). The taxonomy of ascidians reflects the separation of species into vanadium and iron containing species (Hawkins *et al.*, 1983). In the suborders Aplousobranchia and Phlebobranchia, the majority of species' blood contains vanadium. In the suborder Stolidobranchia, vanadium is absent and iron is the major metal in the blood.

Vanadium accumulation by vanadium-containing tunicates has been studied in detail, and some of the steps of vanadium transport have been clearly defined. In sea water, vanadium is present as monomeric vanadate, at a concentration of 6×10^{-8} *M* (Ladd, 1974). Kustin *et al.* (1975) determined that tunicates remove vanadate from the aqueous phase of sea water through the alimentary tract. Once it reaches the tunicate's blood, vanadate is transported through anionic channels into the blood cells (Dingley *et al.*, 1981), where it is reduced (perhaps with the aid of tunichrome) to V(IV) or V(III). It is prevented from leaving the cell because of either its positive charge, VO^{2+}, V^{3+}, or complexation to an organic ligand (Macara *et al.*, 1979; Dingley, 1982).

Unlike that of vanadium, the mechanism of iron uptake by tunicates is unknown. The elementary steps of iron uptake are far from clear even in vertebrates, despite the

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fact that iron is a known, essential trace element. In mammals, for example, two types of iron can be absorbed: heme iron, and nonheme iron. Iron in most food materials is predominantly nonheme iron. It is known that absorption is enhanced in the presence of reducing agents or chelating agents which bind ionic iron. Following iron absorption, its transport in mammals involves specific iron transport proteins (transferrins), and special storage units (ferritins) (Bezkorovainy, 1980). Iron accumulation by iron-containing tunicates has the potential to shed light on vertebrate iron uptake, and is a logical extension in the study of metal accumulation in general.

Unlike vanadium, aqueous iron chemistry is very complex because of extensive hydrolysis, and formation of insoluble hydroxide products. In sea water, iron(III) is the main oxidative species if the marine system is controlled by oxygen (*e.g.*, sea water near the coast; surface waters). Iron(II) is the main species if the marine environment is devoid of oxygen (Kester *et al.*, 1975). Since tunicates generally inhabit well-oxygenated waters, we will primarily be concerned with iron(III) chemistry in sea water.

Styela clava Herdman has a high content of iron in its blood cells (Agudelo *et al.*, 1983). Since iron is a common denominator among the species of the suborder Stolidobranchia, we assume iron to be an essential metal for these ascidians. It is not known what role iron plays in ascidian blood cells, although oxygen transport can be excluded as the blood cell function (Agudelo *et al.*, 1982). In the first paper of this series we described the oxidation state and distribution of iron in *S. clava* and two other iron-containing species.

Little has been done to study the method of uptake or function of iron in tunicate blood. The majority of uptake studies carried out with whole animals have been impaired by iron precipitation (Berg, 1982). Most of the work done on iron accumulating tunicates has been done on the iron-plasma protein association (Hawkins *et al.*, 1980; Webb and Chrystal, 1981), and iron tunichrome interactions *in vitro* (Macara *et al.*, 1979). The present study was undertaken to trace iron uptake by iron-accumulating *Styela clava* from sea water to blood cells.

MATERIALS AND METHODS

Materials

^{55}Ferrous sulfate and ^{55}ferrous citrate were purchased from Amersham Corporation. ^{55}Ferric chloride was obtained from New England Nuclear.

Animals

Specimens of *Styela clava* were obtained from Boston Harbor, at a depth of 1–6 m, and were maintained in a 5°C sea water aquarium. Animals used for uptake experiments varied in size from approximately 5 to 12 cm in height and from approximately 10 to 25 g in weight.

Iron uptake by S. clava *from sea water*

All iron uptake experiments were carried out at 5°C in a plastic container to prevent iron absorption by glass surfaces. *S. clava* specimens were carefully cleaned with a brush and placed in test solutions at time, $t = 0$ (approximately 200 ml of sea water per animal). Aliquots of sea water were removed at timed intervals, for up to 52 hours, and counted using a Beckman LS-100C liquid scintillation counter, to measure the remaining iron in sea water. The rate of uptake was determined from a plot of counts per minute (cpm) *versus* time.

For iron(III) uptake experiments, $^{55}FeCl_3$ was added to sea water along with 1000-fold citrate to prevent precipitation of iron hydroxides. Iron concentrations ranged from 10^{-7}–10^{-6} *M*. Control experiments were run without specimens to insure no iron precipitation. In addition, iron in solution was monitored for several hours before adding the animals. The citrate concentration, added from a neutralized stock solution, never exceeded 5 m*M*. The toxicity of citrate to *S. clava* was checked prior to carrying out the iron(III) uptake studies. Five *S. clava* were placed in 1.66 liters of 0.45 μm filtered sea water in a closed respirometer connected to a YSI oxygen electrode and YSI model 57 oxygen meter. Oxygen consumption was measured for two hours, citrate was then added (total concentration approximately 5 m*M*), and oxygen uptake was measured for an additional two hours.

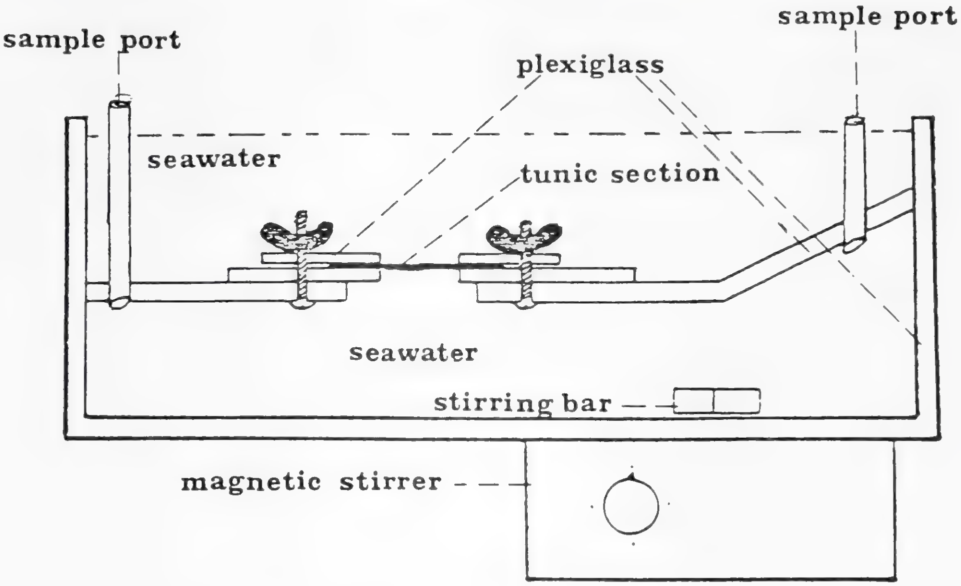
Two additional experiments were conducted to further determine the rate and site of iron(III) uptake. First, the permeability of tunic toward iron was determined using a plexiglass container (Fig. 1) in which pieces of tunic from *S. clava* acted as a partition between two sea water compartments. The outer surface of the tunic (the surface normally exposed to sea water) was exposed to a sea water solution containing radioactive ferric chloride (10^{-7} *M*) and 1000-fold citrate excess. The inner surface of the tunic was exposed to normal sea water (Fig. 1). Sea water samples from each compartment were taken over a two-day period and counted by liquid scintillation.

Second, specimens of *S. clava* were lightly coated with Vaseline to make the surface hydrophobic thereby preventing iron precipitation on the tunic. Animals were then exposed to radiolabeled $FeCl_3$ plus 1000-fold of citrate. Sea water samples were taken at intervals of time over a 52-hour period.

Two iron(II) uptake experiments were carried out. A 20-fold excess of ascorbate was used to maintain the iron in the reduced state. Iron precipitation was not a problem since iron(II) is highly soluble in aqueous solutions. Control experiments were run without specimens to insure no precipitation. In the first experiment, 4 *S. clava* specimens were placed in 600 ml of sea water with 4.4×10^{-7} *M* ^{55}Fe(II)citrate and 0.4 m*M* ascorbate. In the second experiment, two containers were set up, each containing three equal-sized specimens in 500 ml of sea water with 1.0×10^{-7} *M* $^{55}Fe\,SO_4$ and 10^{-6} *M* ascorbate. Sea water aliquots were removed periodically for up to eight hours and counted by liquid scintillation.

Iron uptake by blood cells

The blood of several *S. clava* specimens was collected in a centrifuge tube as previously described (Agudelo *et al.*, 1983). Twenty mg of Cleland's reagent were added to the whole blood to prevent cell agglutination. The blood cells were separated from the plasma by centrifuging at 1200 × *g* for five minutes, and carefully decanting the plasma. A premeasured volume (generally 1.0 ml) of 0.5 *M* NaCl, 0.1 *M* Hepes (pH 7.3) was added to the centrifuge tubes, and the cells were resuspended by vortexing for 30 seconds at a moderate speed. The same amount of buffer was added to another centrifuge tube, as a control. The blood cell suspension and control solution were kept in an ice bath at all times. At time $t = 0$ an equal amount of radioactive iron(II) was added to both the cell suspension and control. Aliquots of blood cell suspension (50–100 μl) were sampled at various intervals of time (approximately every 15 s to 10 min for up to 1 h), transferred into microcentrifuge tubes and centrifuged for 15 seconds at 6500 × *g*. The supernatant was counted by liquid scintillation. An equal volume of control solution was also counted to provide the cpm for supernatant at time zero (initial iron concentration). Since the amount of blood suspension sampled was so small, it would be statistically invalid to count the number of cells in each aliquot.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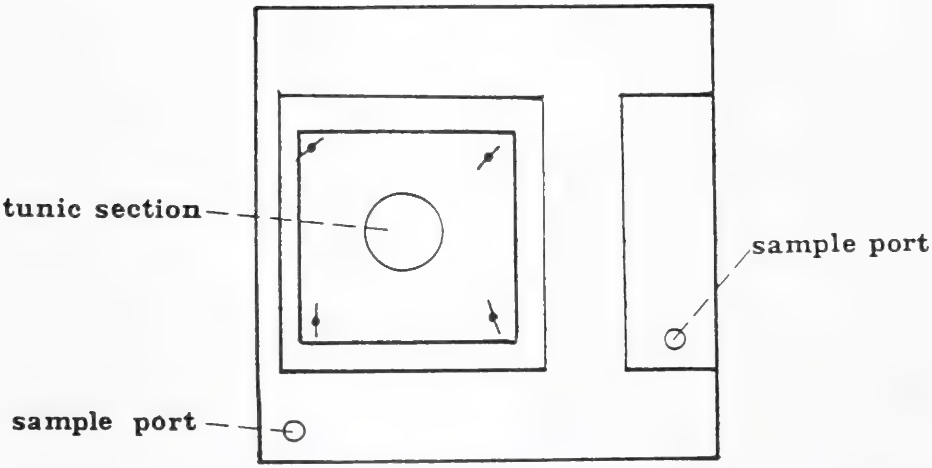
SIDE VIEW


tunic section
sample port
sample port

TOP VIEW

FIGURE 1. Plexiglass container used to determine iron absorption and permeability by *Styela clava* tunic in sea water.

Therefore the decline in iron concentration in the buffer solution is a better indicator of iron uptake by blood cells in suspension.

Several experiments were run to determine the relationship of iron concentration to the cell concentration in the cell suspension. In this case the blood of the specimens was divided into two equal volumes and centrifuged to remove the plasma. One blood pellet was resuspended in buffer to measure iron uptake. The second blood pellet was analyzed for protein content using the Lowry method (Lowry *et al.*, 1951). Protein concentration was taken to be proportional to blood cell concentration.

Several metabolic inhibitors such as citric acid (1–9 m*M*), 2-deoxyglucose (5 m*M*), 4,4-paradinitro-stilbene-2,2 paradisulfonic acid, disodium salt (DNDS) (0.6 mg per ml), and phosphate (2.6 m*M*) were studied. The inhibitor was added to both blank and cell suspensions approximately 15 to 30 minutes before the addition of radioactive iron(II). The effect of an inhibitor was also studied by comparing iron uptake in treated and non-treated cell suspensions. Two blood cell suspensions, with approximately the same number of cells, were placed in an ice bath. The first cell suspension was used as a control; the inhibitor was added to the other suspension. The suspensions were incubated for fifteen minutes before adding the iron. The iron remaining in solution was measured as a function of time, for approximately one hour.

RESULTS

Iron uptake from sea water by S. clava

Citrate concentrations up to 5 m*M* did not have any visible effect on *S. clava* since the rate of oxygen consumption before and after addition of the citrate was the same (Fig. 2). We can therefore assume that there is no acute toxic effect caused by the citrate and that our uptake studies are not affected by the 1000-fold citrate excess used to keep iron(III) from precipitating.

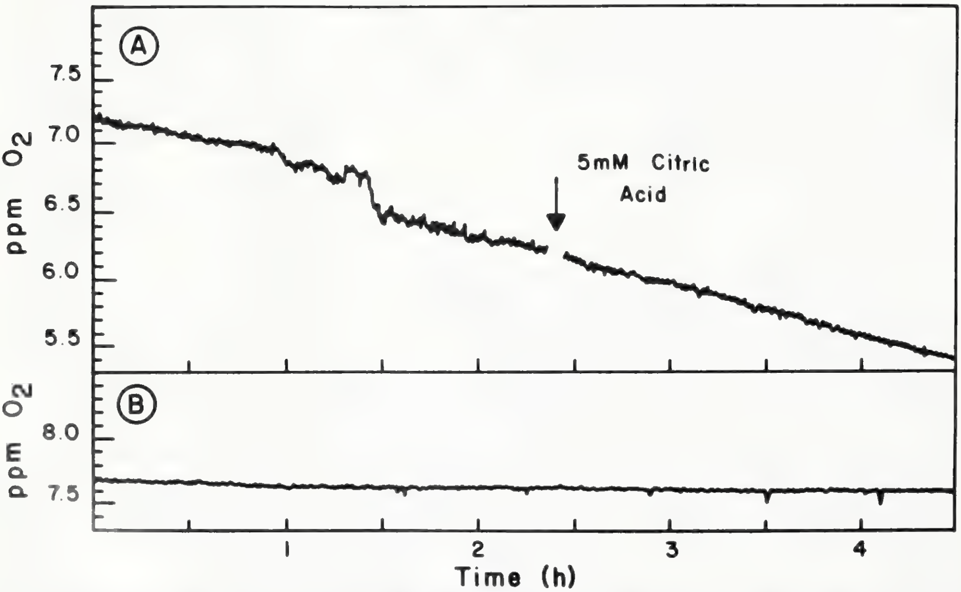


FIGURE 2. Decline in sea water oxygen over time for (A) a respiration chamber containing 5 adult *Styela clava* in 1.66 liters of 0.45 μm filtered sea water (12°C) both before and after the addition of citric acid (final concentration approximately 5 m*M*), and for (B) a respiration chamber filled with sea water alone.

Initial experiments on Fe(III) uptake by intact *S. clava* in solutions containing a 1000-fold excess of citrate demonstrated that iron concentrations in the surrounding sea water declined over time. No decrease in sea water iron concentrations was observed in containers without specimens. Semilog plots of iron cpm *versus* time gave reasonably straight lines with negative slopes for containers with *S. clava*. However, the rate of sea water iron decline varied in each experiment even though the initial iron concentration, the total amount of sea water used, and the size of the animal were approximately the same. Liquid scintillation analyses of tunic pieces dissected from ^{55}iron(III)-exposed animals, showed that the majority of the radioiron was associated with the tunic.

Experiments in which pieces of *S. clava* tunic were used as partitions between two separate chambers (Fig. 1) (one containing radioiron in seawater, the other containing clean sea water) demonstrated that tunic is impermeable to dissolved iron(III) in sea water. Although a decrease in radioactive iron was observed in the sea water exposed to the outer surface of the tunic, no radioiron was found in the sea water exposed to the inner surface of the tunic even after 48 hours of exposure. We conclude that the outer surface of the tunic is responsible for much of the reduction in sea water iron(III) concentrations observed in our previous experiments. However, when a piece of tunic was coated with Vaseline, and placed in sea water with radioiron and a 1000-fold citrate, no decrease of radioiron from solution was observed.

The precipitation of iron(III) onto the tunic was prevented by lightly coating each *S. clava* with Vaseline prior to the start of the experiments. As a result, the decline in sea water Fe(III) concentrations was much slower than observed in previous experiments. The rate of decline was entirely reproducible at 10^{-7} M $^{55}FeCl_3$ (Fig. 3). Although the specimen in container I died within the first six hours of initiating the experiment, iron measurements were continued along with the other two containers for over 36 hours. Remarkably, the rate of radioiron removal from the sea water was the same for all three containers, regardless of whether the animals were dead or alive. These results indicate that there is no active uptake.

In contrast to Fe(III), no uptake of Fe(II) was observed for *S. clava*. Sea water Fe(II)citrate and sea water Fe(II)sulfate concentrations remained constant in both control and experimental containers over the course of 4 to 24 hours.

Iron uptake by S. clava *blood cells*

Iron(II) uptake by blood cells appears to be biphasic (Fig. 4). When the log cpm is plotted *versus* time, the curve obtained can be divided into two linear portions, which we refer to as the fast and slow phases (Table I). Keeping the iron concentration constant, the iron uptake rate in the slow phase varies with the total protein content (*i.e.*, proportional to the number of cells) in the cell suspension. However the calculated rate constants for this phase are the same regardless of the number of cells in the suspension (Table I). The fast phase is not clearly defined probably because of the high concentration of iron used in all three experiments.

If the iron(II) concentration is decreased 10-fold, both the fast and slow phases can be seen more clearly. The rate constant for the slow phase is approximately the same for all experiments ($5 \times 10^{-4} \cdot s^{-1}$). It is clear that the radioiron is taken in by the tunicate blood cells.

Radiolabeled iron(II) was not simply exchanged with iron already present in the blood cells. When the cells were incubated in a medium containing $^{55}FeSO_4$, 44% of the total radioactive iron was taken up by the cell. When the remaining radioactive supernatant was removed by centrifugation and the cells resuspended in clean buffer

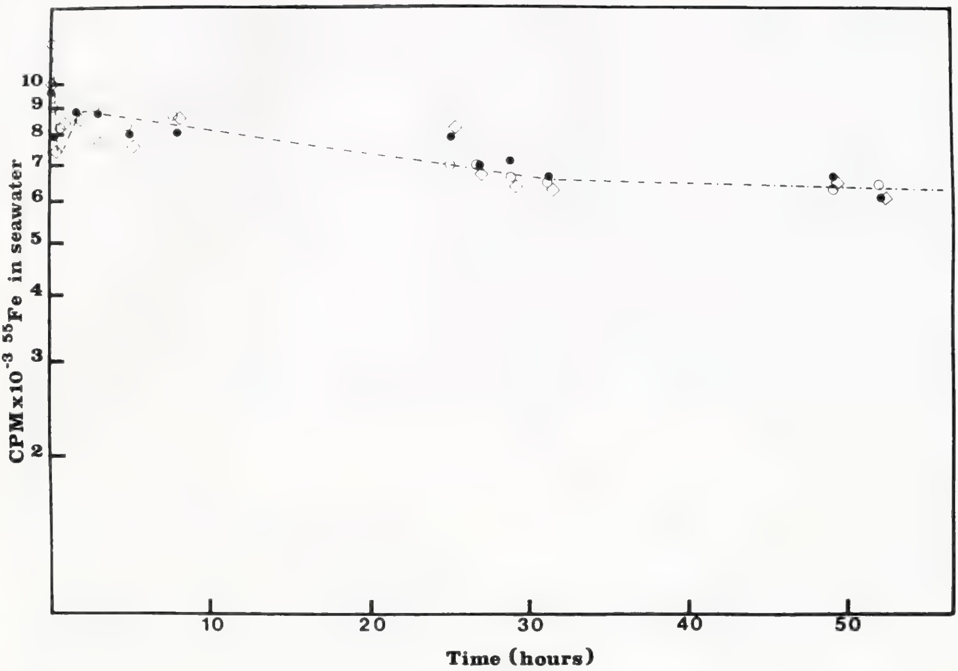


FIGURE 3. Iron uptake by *Styela clava* specimens in sea water containing 1.07×10^{-7} *M* $^{55}FeCl_3$ (specific activity 34.71 mCi mg^{-1} Fe) and 1 m*M* citrate. Container I, ◇, 1 specimen (24.88 g) in 300 ml of sea water; container II, ●, 2 specimens (32.6 g) in 500 ml of sea water; container III, ○, 2 specimens (32.3 g) in 500 ml of sea water.

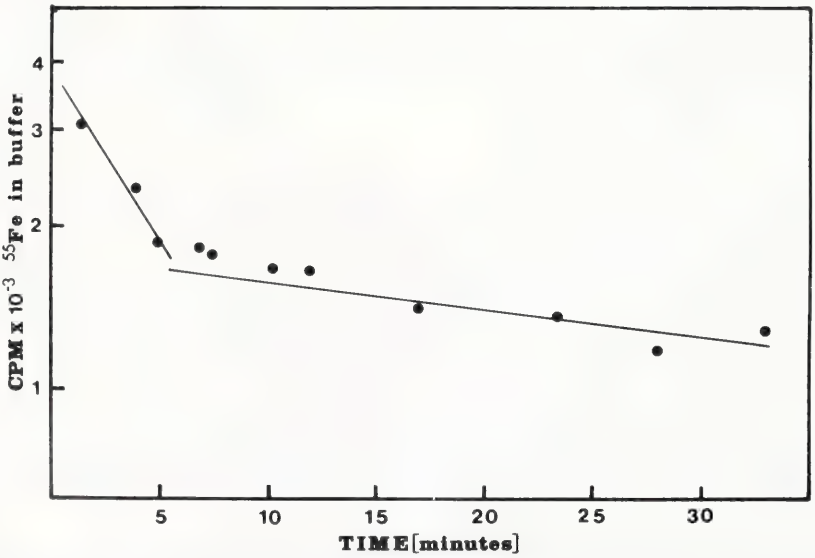


FIGURE 4. Iron(II) uptake by *Styela clava* blood cells suspended in buffer solution (0.1 *M* HEPES, pH 7.3, 0.5 *M* NaCl) containing an initial iron(II) sulfate concentration of 1.6×10^{-6}, and 3×10^{-5} *M* ascorbate. The data was fitted to an exponential function of the form: $y = B_1 + B_2 \exp[-B_3x] + B_4 \exp[-B_5x]$. The constants obtained were: $B_1 = 1130 \pm 9$ cpm, $B_2 = 2907 \pm 38$ cpm, $B_3 = 0.006 \pm 0.0001$ s^{-1}, $B_4 = 1067 \pm 8$ cpm, $B_5 = 0.00058 \pm 0.00002$ s^{-1}.

TABLE I

Iron uptake by Styela clava *blood cells*

Initial Fe(II) conc. (μM)	Total protein conc. (mg)	Rate Constant $\times 10^3 \cdot s^{-1}$ slow
22.1	7.24	0.52
22.1	14.29	0.52
22.1	21.65	0.52

solution, only 2% of the radioiron was observed in solution, probably due to the extracellular buffer solution trapped in the blood cell pellet during centrifugation. The level of radioiron in solution did not increase and therefore no leakage of radioactive iron was observed for 30 minutes. This result indicates that the iron is irreversibly taken up by the cells during the time span in which we studied uptake.

Uptake of Fe(II) was inhibited by 2–9 mM citrate (Tables II and III). Inhibition was not simply due to the drop in pH caused by the addition of citric acid, since inhibition was also observed in neutralized solution. No inhibition of Fe(II) uptake was observed with either 2-deoxyglucose, gallium(3+), phosphate, or the stilbene derivative DNDS (Tables II and III).

DISCUSSION

Iron chemistry in solution is complex, including several solubility products as a result of extensive hydrolysis (Baes and Mesmer, 1976). Iron concentrations quoted for sea water vary greatly (Chester and Stoner, 1974), partly because of the way dissolved and particulate iron are defined. Chester and Stoner (1974) report the dissolved iron concentration in sea water to be $0.4–8.6 \times 10^{-8}$ for near-shore surface waters (water within 400 km of land), having defined dissolved iron as iron that passes through a filter with pores 0.45 μm in diameter, and is retained by a CHELEX column. Betzer and Pilson (1970) report the particulate iron concentration in sea water to be about 3×10^{-9} M, using 0.45 μm filters to retain the particulate matter. These numbers are chosen because the methodologies used are clear. However, it should be noted that many bacteria can pass through 0.45 μm filters. Bacterially associated iron is therefore included in the results obtained by the methods of Chester and Stoner (1974), and Betzer and Pilson (1970).

For iron uptake studies, it is not as important to have an exact number for the total iron concentration in sea water as it is to know what iron species can be found in solution. It is presently impossible to determine either the oxidation state of the

TABLE II

Iron uptake by Styela clava *in the presence of possible inhibitors*

Initial Fe(II) conc. (μM)	Inhibitors	Rate constants $\times 10^3 \cdot s^{-1}$	
		Fast	Slow
1.6	none	6.0	0.58
2.36	citrate (8.7 mM)	no uptake	
2.30	DNDS (1.15 mg)	N.D.*	0.52
6.86	$H_2PO_4^-$ (2.6 mM)	N.D.	1.0

* N.D. = not determined.

TABLE III

Uptake by Styela clava *blood cells in the presence of possible inhibitors* versus *control*

Treatment	Initial Fe(II) conc. in buffer (μM)	Control cells	Treated cells
2-deoxyglucose (5 mM)	2.37	+++	+++
Ga^{3+} (1×10^{-4} M)	2.20	+++	+++
citrate (pH 6.6, 8.7 mM)	2.30	+++	—
citrate (pH 7.3, 2.9 mM)	2.35	+++	—

+++ blood cell iron uptake, — no uptake.

iron, or the major ionic species present in solution at the low concentrations of iron found in sea water. Kester *et al.* (1975) developed a chemical model based on known stability constants and chemical composition of sea water to predict chemical speciation. Based on this model at least 90% of the dissolved iron in oxygenated sea water should be found as iron(III) hydroxide ($Fe(OH)_3$). The remainder is mainly $Fe(OH)_2^+$ with a trace of $Fe(OH)^{2+}$. Since *S. clava* was collected in Boston Harbor at a depth of no more than 6 m, we can assume that oxidation state +3 predominates. However, some of the organically bound iron(III) can be photoreduced by light, creating small concentrations of Fe^{2+} (Anderson and Morel, 1982). According to Kester *et al.* (1975) any ferrous (+2) ion present in sea water at a pH of around 8.0 will be oxidized to iron(III) (95% of it in less than a minute).

Iron uptake by S. clava

There are three sources of iron available to filter-feeding marine organisms: dissolved iron in sea water, particulate inorganic iron, and iron contained in their food source (*e.g.*, phytoplankton and other microorganisms). Results of experiments described in the first section indicate that tunicates either do not actively accumulate iron from the aqueous phase of sea water either as iron(III) or iron(II) or that active iron uptake is so slow that it was not observed in 52 hours of measurements. Therefore, tunicates probably obtain iron either as particulate or colloidal iron [in which case it would be in the form of iron(III) hydroxide], or from their food source.

However it is possible that iron levels in the specimens were sufficiently high so that iron uptake was minimal. Although we attempted to study iron-starved animals, we were unable to maintain animals in synthetic sea water for more than a day. We were therefore not able to restrict the iron diet from sea water using this method.

Investigations of iron uptake in marine invertebrates generally have been limited to the molluscs, particularly the mussel *Mytilus edulis* (Hobden, 1969; Pentreath, 1973; George *et al.*, 1976; George and Coombs, 1977; Lowe and Moore, 1979) and, more recently, the abalone *Haliotis discus* (Tateda *et al.*, 1984). These investigations have centered on the uptake of particulate Fe(III) and Fe(III) organic complexes. Fe(III) uptake by *Mytilus edulis* is primarily by pinocytosis, as evidenced by light microscopical and ultrastructural studies (Fowler *et al.*, 1975; George *et al.*, 1976; George and Coombs, 1977; Lowe and Moore, 1979). This process may occur in a variety of epithelial cells (mantle, digestive gland, kidney, intestine, gill, etc.). Once taken in by pinocytosis, the iron remains within membrane-limited vesicles and may even be transferred in this form to blood cells for subsequent transport throughout the mussel's body. No free cytoplasmic iron has been identified in these epithelial cells, indicating that iron does not cross cell membranes in sufficient quantity to be measured.

The precipitation of particulate $Fe(OH)_3$ onto mucus films may be important for the subsequent uptake of iron by invertebrates. In an autoradiographic study of *Mytilus edulis* tissues, Pentreath (1973) observed that the mucus sheets covering the gills were covered with particulate $^{59}Fe(III)$, whereas ^{65}Zn, being primarily soluble in sea water, was not associated with the mucus coverings. In contrast to these findings, George *et al.* (1976) found no evidence of an iron embedded mucus sheet on *M. edulis* gills at the ultrastructural level. In addition, if the mussel gills were washed, no loss of ^{59}Fe activity was observed, indicating that the label was not extracellular. However, these investigators found that ^{59}Fe was associated with mucus within the stomach and gut. Mucus, whether associated with the gills and stomach of bivalves or lining the branchial basket of tunicates, may collect and hold particulate iron prior to ingestion and subsequent uptake.

Extensive research has been carried out on iron uptake by phytoplankton (Goldberg, 1952; Anderson and Morel, 1982). Anderson and Morel (1982) showed that phytoplankton obtain iron from the free iron concentration in sea water, and that reduction of iron(III) to iron(II) increases iron uptake. They postulated that iron in phytoplankton is probably present in the ferrous state or as an iron(III)-organic complex. They further postulated the presence of a transport protein in the cell membrane which mediates iron uptake from sea water. No conclusive evidence exists on the oxidation state of iron present in this microorganism, nor is there a detailed mechanism of iron uptake. These facts must be known before a definitive study of iron uptake by tunicates from microorganisms can be undertaken.

Blood cell iron uptake

There is still a large gap in our knowledge between iron uptake by the tunicate from sea water, and iron uptake by tunicate blood cells. We can only postulate that iron is obtained by digestion of particulate matter (colloidal iron, microorganisms) in the digestive tract, where it is solubilized into a form that will reach the blood plasma.

Because of the low iron concentration in the plasma, it is difficult to specify the oxidation state of the iron present. There are proteins in the plasma that are capable of binding iron (Hawkins *et al.*, 1980). Furthermore, iron inside the blood cells is present as iron(II), and 70% of this iron is associated with cell membranes, (Agudelo *et al.*, 1983). From these data, we can hypothesize that there may be a transferrin-like protein present in the blood cell membranes that transports iron into the cells.

Tunicate blood cells accumulate iron(II) from the suspending medium. Two steps are observed: the first is probably the rapid binding of the iron(II) to the surface membrane of the cell; the second is slower transport of iron into the cell. This is the most likely mechanism, since the first step depends on iron concentration (although not enough data has been obtained to find the relationship of rate *versus* Fe concentration), while the slow step is independent of iron concentration.

Citrate completely inhibits iron(II) uptake by blood cells. This effect is probably due to complexation of iron(II), making it unavailable to the membrane proteins of the cell. Another possibility is that citrate can enter the cytosol (probably through an anionic channel), where it can inhibit phosphofructokinase. Excess citrate leads to a decrease in the rate of glycolysis (Lehninger, 1975), thereby preventing iron uptake, if it is an active process.

2-Deoxyglucose is carried into the cell by the glucose carrier (Lehninger, 1975). However, it will slow the rate of glycolysis because it can not be metabolized like glucose. 2-Deoxyglucose would thus have a similar effect on iron(II) uptake as citrate, if the process is an active one. When added to the cell suspension, 2-deoxyglucose showed no effect on iron(II). Therefore, we can conclude that iron(II) uptake is not an active process.

Although the uptake experiments show that the iron(II) goes into the blood cells, they do not tell us anything about whether the iron is oxidized before or during the process of entering the cell. Gallium has been used as a substitute for iron in many iron metabolism studies (Emery, 1982). Ga^{3+} and Fe^{3+} ions are very similar in size (0.062 nm and 0.064 nm, respectively) and carry the same charge. If the uptake process requires iron(III) in any step, by incubating with gallium(III) prior to the uptake studies, inhibition or a decrease in the rate should be observed since a significant number of binding sites would be occupied by gallium. That gallium does not affect iron(II) uptake implies that there is no oxidation step in the iron(II) uptake by the blood cells. This result does not preclude the possibility that iron(III) could be transported into the cells. In this case it is possible that the first step would require iron(III) being reduced to iron(II). Iron(III) uptake studies by blood cells were not addressed because of the low solubility of iron(III). At this point, due to interference by precipitation, iron(III) uptake studies would be ambiguous, and firm mechanistic conclusions could not be drawn from them.

In summary, iron taken up by *S. clava* is most likely in particulate form, or incorporated in microorganisms available as food.

Once the iron reaches the plasma, it can be accumulated by the blood cells if it is in the iron(II) form, without any steps requiring oxidation changes. Because it is inhibited by citrate, iron(II) must be in its ionic form. Iron(II) uptake is an irreversible process. No conclusions can be reached about iron(III) uptake by the blood cells.

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SULFIDE OXIDATION OCCURS IN THE ANIMAL TISSUE OF THE GUTLESS CLAM, *SOLEMYA REIDI*

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ABSTRACT

A gutless clam, *Solemya reidi,* from sulfide-rich habitats has gills containing symbiotic, chemoautolithotrophic bacteria that are presumed to oxidize sulfide to provide a major energy source for the symbiosis. Sulfide oxidation was studied for *S. reidi;* activity of gill and foot extracts displayed Michaelis-Menten kinetics and was presumably due to sulfide oxidase enzymes. The activity of *S. reidi* gill extracts was protease sensitive and heat sensitive and was 10 to 20 times higher than in tissues from bivalves not living in sulfide-rich environments. The site of sulfide oxidation was studied by cytochemistry, ^{35}S-sulfide autoradiography, cell fractionation methods, and by X-ray microanalysis. In gills of *S. reidi,* the sulfide-oxidizing activity was detected not in the symbiotic bacteria, but within organelles of the gill cells we have named sulfide oxidizing bodies. In foot tissue of *S. reidi,* sulfide oxidation activity was distributed diffusely throughout the superficial cell layers of the foot. The discussion considers the roles of sulfide oxidation in protection of aerobic respiration from sulfide inhibition and in exploitation of the energy contained in sulfide.

INTRODUCTION

A variety of marine invertebrates (pogonophoran tube worms, bivalve molluscs, and oligochaetes) from sulfide-rich habitats harbor sulfur bacteria as symbionts (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981; Cavanaugh, 1983; Felbeck *et al.*, 1983; Fisher and Hand, 1984). These bacteria have enzymes of the Calvin-Benson cycle (Felbeck, 1981; Felbeck *et al.*, 1981; Felbeck and Somero, 1982; Felbeck, 1983; Felbeck *et al.*, 1983; Fisher and Hand, 1984), and the energy (reducing power and ATP) needed for net CO_2 fixation, as well as for reducing nitrate and nitrite, is thought to come from the exergonic oxidation of sulfide to, *e.g.*, sulfate (*cf.* Jannasch, 1984). Enzymes involved in ATP- and NAD(P)H-generating sulfide oxidation pathways have been found in these symbioses (*cf.* Felbeck *et al.*, 1981).

In addition to possessing the enzymic pathways for a sulfide-based chemoautolithotrophic type of metabolism, these symbioses are likely to depend on a second important type of adaptation to sulfide, mechanisms which prevent the poisoning of aerobic respiration by sulfide. Micromolar amounts of hydrogen sulfide are extremely toxic to most organisms because it inhibits the cytochrome c oxidase system (National Research Council, 1979). Yet in the habitats where these symbioses have been found, sulfide concentrations range between about 100 μM and 20 mM (Edmond *et al.*, 1982; Felbeck, 1983). Because levels of cytochrome c oxidase activity in these symbioses and in related species from sulfide-free habitats are similar, it appears that these symbioses are able to carry out aerobic respiration (Hand and Somero, 1983; Powell and Somero, 1983).

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Our initial study of the mechanisms that prevent poisoning by sulfide of aerobic respiration focused on the hydrothermal vent vestimentiferan tube worm, *Riftia pachyptila* (phylum Pogonophora) (Powell and Somero, 1983). We showed that the respiration of obturacular plume tissue in the presence of high concentrations of sulfide was not due to an inability of sulfide to penetrate this tissue or to a sulfide-insensitive form of cytochrome c oxidase. Instead, we showed that a component of the worm's blood, probably the sulfide binding protein (Arp and Childress, 1983), was responsible for protecting cytochrome c oxidase activity. The sulfide binding protein does not function as a sulfide oxidase. Instead, it binds sulfide very tightly, and acts as a sulfide transport mechanism (Arp and Childress, 1983) and as a means for preventing sulfide concentrations in the animals' cells from reaching levels inhibitory to aerobic respiration (Powell and Somero, 1983).

Despite the importance of the sulfide binding protein for protecting aerobic respiration from sulfide in *Riftia pachyptila,* we reasoned that other means for keeping free sulfide from contact with cytochrome c oxidase are also apt to be critical for animals of sulfide-rich habitats. For example, cells of poorly perfused tissues may not have an adequate amount of blood-borne sulfide binding protein to complex the sulfide entering the tissue. Also, not all animals inhabiting sulfide-rich habitats possess sulfide binding proteins (A. J. Arp and J. J. Childress, pers. comm.). In these cases, it may be essential for the animal's cells to convert sulfide to a non-toxic sulfur compound. In fact, even in tissues with symbionts it is unclear how sulfide from the blood or seawater is provided to the bacteria without the concomitant inhibition of mitochondrial respiration in the symbiont-containing cells. Do enzyme systems in the animal cytoplasm first detoxify sulfide and, then, transport to the bacteria a partially oxidized form of sulfur?

Because of the uncertainties about how and where sulfide is metabolized in these sulfide biome symbioses, we studied sulfide metabolism in the clam, *Solemya reidi.* This animal is a gutless, protobranch bivalve which inhabits sulfide-rich zones like sewage outfall areas (Felbeck, 1983) and pulpmill effluent sites (Reid, 1980). It contains large numbers of bacterial symbionts in its gill, where enzymes of sulfur metabolism and the Calvin-Benson cycle are found (Felbeck, 1983); it has high levels of sulfide-sensitive cytochrome c oxidase activity in gill and foot tissues (Hand and Somero, 1983, unpub. obs. of authors); and it lacks a circulating sulfide-binding protein (A. J. Arp and J. J. Childress, pers. comm.).

Our results suggest that in *S. reidi* gills the initial steps in sulfide oxidation occur in specific organelles in the gill cytoplasm, sulfide oxidizing bodies, not in the bacteria. The implications of this localization of sulfide oxidizing activity for the exploitation of the energy contained in sulfide are discussed. In foot tissue, which lacks bacteria, sulfide oxidizing activities are restricted to the superficial cell layers of the foot muscle. The sulfide oxidizing activities of gill and foot display diagnostic characteristics of enzymes. In both tissues these sulfide oxidizing activities may play important roles in protecting the aerobic respiration of the clam from poisoning by sulfide.

MATERIALS AND METHODS

Animal collection

Collection of *S. reidi* was by grab sampling of sewage outfall mud near White's Point, California, at a depth of about 100 m. *Mercenaria mercenaria* were purchased from Pt. Loma Seafoods, San Diego, CA. *Chione sp.* were collected from mud flats in Mission Bay, San Diego, CA. *Mytilus edulis* were collected intertidally near Scripps

Institution of Oceanography, La Jolla, CA. Specimens of the teleost fish, *Sebastolobus altivelis*, were collected by trawling, and frozen immediately on dry ice. *S. reidi* were maintained in mud taken from their site of capture, with 8°C seawater slowly flowing over the mud. The other invertebrate species were maintained in flowing seawater at ambient local seawater temperatures (14–18°C). Most experiments were performed with tissues taken from freshly killed animals maintained in the lab less than one month. Animals frozen immediately after capture were used for a few experiments.

Sulfide solutions

Sulfide solutions (usually 100 m*M*) were made fresh using crystals of Na_2S washed in distilled H_2O to remove oxidized material on the surface, and used within several hrs. The term "sulfide" refers to all forms of sulfide present in solution, H_2S, HS^-, and S^{2-}.

Tissue preparation

Tissue samples (1:10 tissue:buffer in most cases; 1:4 when activity was low) were homogenized on ice in 50 m*M* Tris/HCl pH 8.0 (20°C), containing 0.1% Triton X-100. Some homogenates were used uncentrifuged and when clear samples were required (for the spectrophotometric assays) homogenates were centrifuged 10 min at 10,000 × *g*.

Measurement of sulfide oxidation

Disappearance of sulfide was monitored using a modification of the procedure of Moriarty and Nicholas (1969) with an Orion sulfide-sensitive solid-state microelectrode and microreference electrode connected to an Orion pH/ion electrode meter. The reaction mixture contained 0.80–0.99 ml 40 m*M* glycine buffer pH 9.0, 0–0.1 ml 100 m*M* Na_2S, and 0–0.1 ml sample. All measurements were made aerobically with stirring. The electrode was standardized with solutions of sulfide in 40 m*M* glycine pH 9.0, and rates of sulfide disappearance in the absence of tissue homogenates were measured at various sulfide concentrations. The addition of tissue homogenates shifted the standardization of the electrode so the following procedure was used for measuring sulfide disappearance in the presence of tissue homogenates. First, tissue homogenates were added to the reaction chamber while the buffer was stirred. Second, sulfide was added to the stirred mixture and the initial reading was used as a point in the standardization of the electrode in the presence of tissue homogenates. Third, the decline in sulfide concentration was recorded. This procedure was followed for a number of sulfide concentrations, thus generating a new standard curve for the electrode in the presence of the tissue homogenate being used. Control rates (no homogenate) of sulfide disappearance were subtracted from rates in the presence of tissue homogenates to yield the tissue-specific rates of sulfide oxidation.

The second method used to measure sulfide oxidation involved reduction of the artificial electron acceptor benzyl viologen. Benzyl viologen is colorless when oxidized and intensely purple when reduced. It is rapidly reoxidized by oxygen, so assays must be performed anaerobically. Sulfide levels below 5 m*M* reduce benzyl viologen very slowly in the absence of added tissue homogenates. Higher levels of sulfide lead to an increase in the rate of the non-enzymic reaction, so rates of sulfide oxidation caused by tissue homogenates are best measured using sulfide levels of 5 m*M* and below. The reaction mixture contained 40 m*M* glycine buffer pH 9.0, 2 m*M* benzyl viologen, 0–5 m*M* Na_2S, 0–200 μl sample in a total volume of 1 ml. Reduction of benzyl viologen was followed at 578 nm using an extinction coefficient of 8.65 absorbance units × mM^{-1} × cm^{-1} (McKellar and Sprott, 1979).

Protease treatment

Protease treatment was done at 37°C for 21 h. The reaction mixture consisted of gill extract in 50 m*M* Tris-HCl pH 8.0, 0.1% Triton X-100, and one of the following proteases: pronase (0.5 mg, 4 casein units), trypsin (2.0 mg, 20,000 BAEE units), or chymotrypsin (2.0 mg, 100 BTEE units) in a total volume of 0.1 ml. Proteases were purchased from Sigma Chemical Co.

Activity staining of acrylamide gels

Gels of 7 and 10% acrylamide with 0.1% Triton X-100 added were stained using a modification of the procedure of Jacobson *et al.* (1982) for staining gels of hydrogenase using H_2 and methyl viologen. The gel was carefully inserted into a Ziploc bag and a solution containing 0.2 *M* glycine pH 9.0, 5 m*M* Na_2S, and 2 m*M* benzyl viologen was added. Bubbles were removed, the bag was sealed, and purple colored bands developed in 5–20 min.

Cytochemistry

Sections 6–9 μm thick were cut from unfixed tissue frozen at −70°C in Tissue-Tek II O.C.T. compound and from tissue fixed in 3% glutaraldehyde in 77% seawater for 2 h at room temperature prior to freezing. Sections were stained by addition of 0.2 *M* glycine pH 9.0, 5 m*M* Na_2S, 2 m*M* benzyl viologen made immediately before use. A drop of staining solution was applied to the section and a cover slip was added immediately. Purple color developed in less than 5 min in positively staining areas.

Sections were frozen by addition of a layer of distilled H_2O to sections and insertion of slides into a −20°C freezer until thoroughly frozen. Slides were then thawed at room temperature until no ice was visible.

Toluene treatment consisted of immersion of the section into toluene for several rinses of several min each, followed by an ethanol rinse.

^{35}S-sulfide autoradiography

Clams were used for autoradiography within 30 min of capture. Incubations and tissue fixations were done at sea on board the research vessel R/V Velero IV. The autoradiography buffer consisted of seawater buffered with 20 m*M* HEPES, pH 7.5. Isolated gills were used for the 1 and 10 min incubations, and intact live clams for the 1-h incubation.

The 1-min experiment consisted of isolated gills placed in one ml of buffered seawater at 10°C containing 60 μCi of ^{35}S-sulfide and 1 m*M* total sulfide. After one min the gills were rapidly rinsed twice in 50 ml 10°C buffered seawater lacking sulfide, blotted to remove excess liquid, placed in a foil boat containing sectioning medium, and frozen in liquid N_2 for later sectioning. The total time elapsed between removal from labeled sulfide and freezing was less than 1 min.

The 10-min experiment followed the same procedure except 10 μCi of sulfide were used and the incubation was for 10 min.

The 1-h experiment consisted of small (less than 1 cm) intact live clams incubated in 1 ml of buffered seawater containing 10 μCi of ^{35}S-sulfide and 1 m*M* total sulfide. The valves remained open for most of the incubation period. After 1 h, the clams were rinsed briefly twice in 10°C buffered seawater lacking sulfide and the gills were removed rapidly, rinsed again twice, blotted to remove excess liquid and frozen as above. Total time elapsed between removal from labeled sulfide and freezing was less than 5 min.

The tissue was frozen for sectioning rather than fixed and embedded because we believed fixation and embedding would be so slow as to allow the diffusion and metabolism of sulfide to continue after the 1-min incubation was over; also, the fixation and embedding process might cause loss of labeled metabolites from the tissue. Four days later, after returning to port, tissues were cut into 6–9 μm sections in a darkroom under a safelight compatible with autoradiography emulsion. Sections were picked up onto Kodak NTB-2 coated slides, placed into slide boxes with desiccant and allowed to expose at 4°C for 8 days. The emulsion was then developed and the slides were mounted.

Isolation of sulfide oxidizing bodies

Gill tissue was homogenized 1:10 in 20 m*M* HEPES pH 7.5, 0.4 *M* sucrose, 1 m*M* EGTA, and 0.5% BSA using a Polytron mechanical homogenizer at ½ speed for 3 bursts of 5 s each with 15 s between bursts to prevent heating of the sample. The sample tube was immersed in an ice and water bath during the entire operation. The sample was then homogenized on ice using a Dounce glass homogenizer, first with a loose, then a tight pestle. Care was taken to avoid foaming and the formation of a vacuum below the pestle. Homogenization was judged to be complete when few intact cells were observed under a microscope.

The sample was then diluted 1:5 with 20 m*M* HEPES pH 7.5, containing 0.4 *M* sucrose and filtered by gravity through a 49 μm Nytex filter with gentle stirring in an Amicon ultrafiltration apparatus. The resulting filtrate was then centrifuged 10 min at 10,000 × *g*, and the resulting pellet was rehomogenized gently with a Dounce glass homogenizer in a volume of 60% Percoll in HEPES/sucrose equal to about a 1:10 dilution of the original tissue weight.

The mixture was then centrifuged 120 min at 25,000 × *g* at 4°C in a fixed angle rotor (Sorvall SS-34) to generate a gradient and separate the sample components simultaneously. 0.45-ml fractions were collected from the bottom of the gradient using a peristaltic pump. A tight dark brown band formed near the bottom of the gradient and a chalky brown band formed near the top. Gradients were not monitored for density.

The sulfide-oxidase activity of the fractions was measured. All fractions with substantial sulfide oxidase activity and at least every fourth fraction were counted for bacteria and sulfide oxidizing bodies using a hemocytometer. The counting was simplified by the large size (3–10 μm) and characteristic rod shape of the bacteria. Round structures with a brown-yellow color and 2–5 μm in diameter were counted as sulfide oxidizing bodies. The few nuclei present were clearly distinguishable from sulfide oxidizing bodies.

Microscopy

Tissues were taken from animals within 30 min of capture, and fixed in freshly made 3% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.35, containing 0.35 *M* sucrose, at room temperature for 90 min, and stored at 4°C. After returning to port (4 days after collection), tissues were rinsed 3 times in phosphate/sucrose buffer lacking glutaraldehyde and postfixed for 1 h in 1% OsO_4 in phosphate/sucrose at 0°C. Tissues were then rinsed in distilled H_2O, dehydrated through graded ethanol solutions, and embedded in Spurr's resin.

Samples used in X-ray microanalysis were not postfixed in OsO_4 because it caused decreased signal to noise ratio during analysis for sulfur.

Sections for light microscopy were cut to 1 or 2 μm, mounted on slides, and stained. Sections for electron microscopy were cut to a thickness of 80–90 A, and stained with uranyl acetate and lead citrate. Sections for X-ray microanalysis were cut to 0.2–1.0 μm, picked up on carbon coated nylon grids, coated with carbon, and used unstained.

RESULTS

Detection of sulfide-oxidizing activity

The two techniques we used for measuring sulfide oxidation, monitoring sulfide disappearance with a sulfide electrode (Moriarty and Nicholas, 1969), and a more sensitive technique which follows the reduction of the artificial electron acceptor benzyl viologen, yielded similar values for the sulfide oxidizing capacity of the tissues we examined. Table I gives data for gill tissue of *Solemya reidi*, the tissue we found to contain the highest amounts of sulfide-oxidizing activity. The variation between assay methods was less than 25%. Since initial studies comparing the two methods showed similar levels of activity, and the benzyl viologen method was much more sensitive and easier to perform, further experiments were conducted using only the benzyl viologen reduction method.

Rates of sulfide oxidizing activity

Because sulfide oxidation occurs spontaneously (non-enzymatically) in seawater containing oxygen, and because metal ions and proteins have been found to stimulate sulfide oxidation, any solution containing tissue extract is liable to have some capacity for oxidizing sulfide (Baxter and Van Reen, 1958; Sorbo, 1960; Chen and Morris, 1972; Almgren and Hagstrom, 1974). Thus, to determine whether the activities found in gill and foot of *S. reidi* were substantially higher than activities present in tissues of animals from sulfide-free habitats, we compared rates of sulfide oxidation of assay mixtures containing tissue extracts from *S. reidi* with those found for extracts from tissues of other species and with solutions of known types of proteins (Table II). Tissues from *S. reidi* had the highest levels of activity, especially the gill. The foot tissue of *S. reidi* had a capacity for oxidizing sulfide that was significantly higher (*t*-test, $P = 0.05$) than that of the other bivalve studied, *Mercenaria mercenaria*. Homogenates of the

TABLE I

Sulfide oxidation activity in Solemya reidi *gills*

Sample	Spectrophotometric assay (Units × gFW^{-1})	Sulfide electrode assay (Units × gFW^{-1})
1	2.3	3
2	6.9	7.6
3	8.1	10

Sulfide oxidation activity was measured in *Solemya reidi* gill homogenates using two different assay techniques (details in methods). Activity is expressed in international units (μmoles of substrate converted to product per min) per gram fresh weight of tissue at 20°C.

TABLE II

Sulfide oxidizing activity

Sample	Units × gFW^{-1}	Units × ng protein^{-1}
S. reidi		
gill	6.38 ± 3.47 (9)	64.4 ± 35.0 (9)
foot	0.38 ± 0.07 (6)	5.3 ± 0.7 (6)
foot surface	0.88 (1)	12 (1)
M. mercenaria		
gill	0.33 ± 0.06 (2)	4.7 ± 0.2 (2)
foot	0.22 ± 0.04 (2)	2.7 ± 0.1 (2)
foot surface	0.23 (1)	4.4 (1)
adductor	0.23 ± 0.03 (2)	4.3 ± 1.4 (2)
mantle	0.20 ± 0.05 (2)	2.9 ± 1.3 (2)
S. altivelis		
muscle	0.26 (1)	1.7 (1)
Protein solutions		
albumin (bovine serum)		1.4
trypsin		1.3
myoglobin		1.2
chymotrypsin		0.9
RNAse A		0.9
poly-1-lysine		0.3

Sulfide oxidation activity of tissue supernatants and protein solutions measured using the spectrophotometric assay. Values expressed as mean ± standard deviation (number of individuals). Protein concentration in assay mixtures of protein solutions was 1 mg/ml (n = 1 for protein solutions).

entire foot failed to give a true picture of the foot's sulfide oxidation potential, however. Cytochemical staining (see below) revealed that the surface layer of the foot of *S. reidi* was where most of the sulfide oxidizing activity was found, so the real ability of the foot to oxidize (detoxify) sulfide is better indicated by the activity obtained using homogenates of tissue taken from the surface of the foot. Cytochemical staining of the other bivalves studied failed to reveal surface layers of activity. For foot of *S. reidi,* the surface layer contained sulfide oxidizing activity up to twice those of whole foot homogenates. For foot of *M. mercenaria,* the single experiment comparing whole foot *versus* foot surface activities showed no difference in rates. White skeletal muscle tissue of the marine teleost fish, *Sebastolobus altivelis,* had roughly the same sulfide oxidizing capacity as the tissues of *M. mercenaria.*

With our benzyl viologen assay system, approximately 0.25 units of sulfide oxidizing activity per g fresh weight of tissue appear to reflect a common, basal level of sulfide oxidizing activity in many animal tissues. This basal level of activity could reflect relatively non-specific catalysis of sulfide oxidation by proteins and metal ions. All of the purified proteins studied gave measurable rates of sulfide oxidizing activity (Table II). These rates were the lowest rates of sulfide oxidation found, but, assuming a tissue protein content of approximately 150 mg protein per g fresh weight of tissue, the predicted rate of sulfide oxidation due to "protein" would be about 0.2 units per g fresh weight of tissue.

Evidence for an enzymatic basis of sulfide oxidation in S. reidi

The sulfide oxidizing activity in extracts of *S. reidi* foot and gill tissue exhibited Michaelis-Menten kinetics (Fig. 1). The K_m of gill extract was 1.01 ± 0.08 (S.E.). The

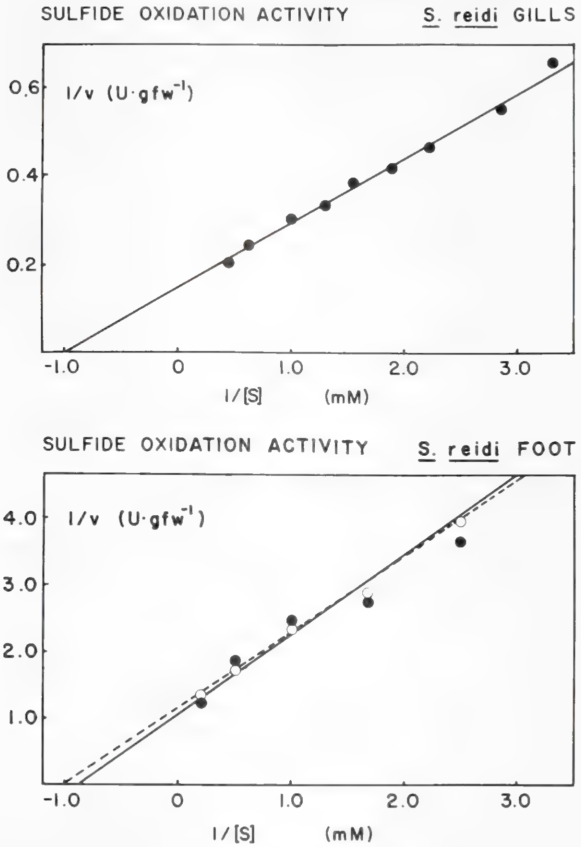

FIGURE 1. Double-reciprocal (Lineweaver-Burk) plots of sulfide oxidation activity of *Solemya reidi* gill (Top) and foot (Bottom) supernatants. Activities are expressed as international units per g fresh weight ($U \cdot gFW^{-1}$). For Figure 1B (Bottom), open circles and dashed line (– – –) represent 8.3 mg fresh weight used per assay, and closed circles and solid line (——) represent 4.2 mg fresh weight used per assay. Best fit straight lines were calculated using a weighted linear regression program.

K_m of an extract prepared from the surface of foot tissue was 1.00 ± 0.17 when 8.3 mg fresh weight were used and 1.20 ± 0.41 when 4.2 mg fresh weight were used. Protease treatment of gill supernatant from *S. reidi* resulted in up to an 80% loss in sulfide oxidizing activity, and boiling for 30 min caused a 50% loss of activity (Table III). Electrophoresis of *S. reidi* gill homogenates on native acrylamide gels, followed by sulfide oxidation activity staining, resulted in a single band in the separating gel exhibiting activity, as well as a band at the interface between the stacking and separating gel (data not shown). These results suggest that the sulfide oxidizing activity in *S. reidi* is due to the activity of one or more "sulfide oxidase" enzyme systems.

In contrast to the results obtained with extracts of tissues from *S. reidi*, the sulfide oxidizing activities of the protein solutions and the tissue extracts of animals from low-sulfide habitats showed no evidence of substrate (sulfide) saturation at sulfide concentrations up to 10 m*M* (the highest concentration compatible with the benzyl viologen assay system). These sulfide oxidizing activities appear not to be due to a specific "sulfide oxidase" enzyme having Michaelis-Menten kinetics.

The factor responsible for the activity is probably either membrane bound or enclosed within membranes; inclusion of 0.1% Triton X-100 in the homogenization

TABLE III

Susceptibility of sulfide oxidizing activity to protease treatment and boiling

Sample	Units × gFW^{-1}
Untreated *S. reidi* gill	3.3
S. reidi gill, no protease (21 h, 37°C)	1.4
S. reidi gill + chymotrypsin (21 h, 37°C)	0.65
S. reidi gill + trypsin (21 h, 37°C)	0.53
S. reidi gill + pronase (21 h, 37°C)	0.60
S. reidi gill, boiled (30 min)	1.7

Sulfide oxidation activity was measured using the benzyl viologen assay. Decrease in activity at 37°C without added protease may be due to endogenous proteases.

buffers resulted in increased levels of sulfide oxidizing activity in 10,000 *g* × 10′ supernatants, and inclusion of 0.1% Triton X-100 in electrophoresis buffers and gels caused more of the activity to enter the separating gel.

Cytochemical localization of sulfide-oxidation activity

Since the gill homogenates contained the highest levels of sulfide oxidation activity, and the gills contained vast numbers of endosymbiotic bacteria, we wished to test the

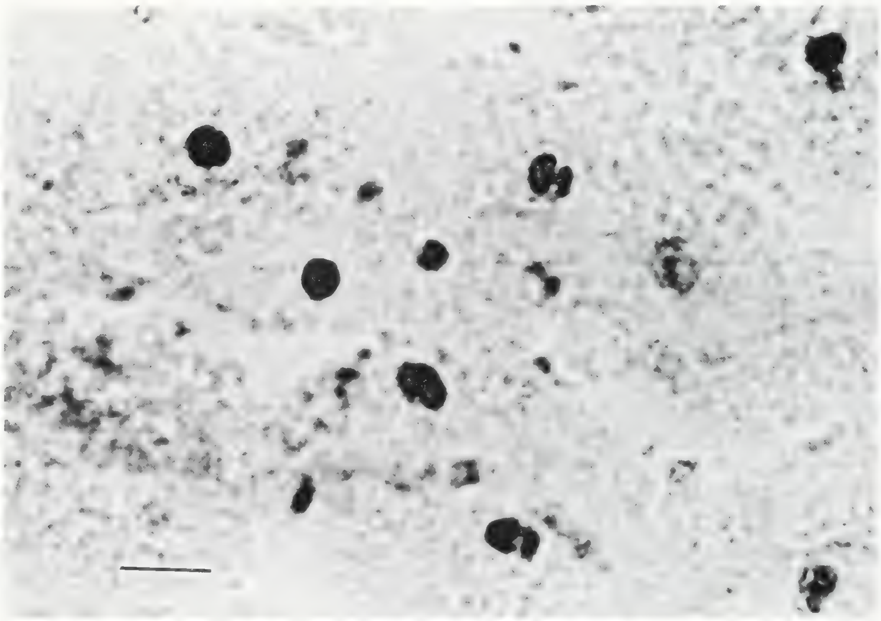

FIGURE 2. Sulfide oxidation activity staining of 6 μm section of *Solemya reidi* gill. Scale bar = 10 μm. The dark structures about 2–5 μm in diameter are the positively stained sulfide oxidizing bodies.

hypothesis that the bacteria are the site of sulfide oxidation. We modified the benzyl viologen sulfide oxidation assay to work as a cytochemical stain (see Materials and Methods). Unfixed tissue freshly cut in a cryostat was incubated with sulfide and benzyl viologen anaerobically (*i.e.*, under a coverslip). Cytochemical staining with sulfide and benzyl viologen resulted in the formation of purple color at the sites of sulfide oxidation (Fig. 2). No color was formed when benzyl viologen was added without sulfide. Staining of fixed tissue failed to show any sites of sulfide oxidation, and staining of sections cut from gill and foot tissue from two other marine bivalves, *Mytilus edulis* and *Chione* sp., also failed to show sulfide oxidizing activity (data not shown).

The bacteria in the gills of *S. reidi* did not stain positively for sulfide oxidizing activity. Rather, staining was observed exclusively in the round organelles we term sulfide oxidizing bodies, which ranged in diameter from about 1 μm to 10 μm. The sulfide oxidizing bodies appeared yellow-brown in unstained sections. To ensure that the lack of positive staining for sulfide oxidizing activity in the bacteria was not due to a failure of the sulfide or staining reagents to penetrate the bacteria, we treated some gill sections with toluene (Harwood and Peterkofsky, 1975) and subjected others to freezing and thawing so as to increase the likelihood that the bacteria were freely permeable (see Materials and Methods). Neither treatment led to a positive sulfide oxidase stain in the bacteria, but the sulfide oxidizing bodies stained positively after both treatments.

Isolation of sulfide oxidizing bodies

Table IV shows the result of density gradient centrifugation of *S. reidi* gill homogenates, and the sulfide-oxidation activity of the resulting fractions. Sulfide-oxidation activity is found only in those fractions observed to contain sulfide-oxidizing bodies, and the sulfide-oxidation activity peaks were found in the same fractions as the greatest numbers of sulfide-oxidizing bodies. Fraction 2 contained the most highly purified sulfide-oxidizing bodies; it was about twenty-five-fold enriched in these bodies and eighty-fold enriched in sulfide oxidation activity, and about forty-fold depleted in bacteria (relative to the initial tissue homogenate prior to centrifugation). Sulfide ox-

TABLE IV

Sulfide oxidizing body isolation

Fraction	Sulfide oxidizing bodies (#/ml, × 10^6)	Bacteria (#/ml, × 10^6)	Sulfide oxidizing activity (units × μl^{-1})
1	2.8	0.7	88
2	330	0.1	3480
6	21	3.4	51
10	13	4.6	37
14	9.6	27	69
16	140	140	560
17	2.2	6.0	102
Initial homogenate	13	3.9	45

Results of representative Percoll density gradient isolation of sulfide oxidizing bodies. Fraction 1 was the bottom of the gradient. Most of the sulfide oxidizing bodies formed a dense band (fraction 2) well separated from other particulate constituents of the sample (fraction 16). Fractions 2 and 16 contained the only visible bands of particles in the gradient. Fractions not shown contained low sulfide oxidizing activity.

idizing bodies were found to be very dense particles, a fact which contributed to their simple isolation. The procedure used essentially sediments most of the sulfide oxidizing bodies away from all other particles.

^{32}S-sulfide autoradiography

^{35}S-sulfide autoradiography was performed to localize sites of sulfide metabolism. Figure 3 shows the results of exposure to ^{35}S-sulfide for 1 min.

The major concentration of ^{35}S was in the region of the gill where the clam cells contained sulfide oxidizing bodies but lacked bacteria (Region 2, Fig. 4). Longer incubations in ^{35}S-sulfide (isolated gills for 10 min and intact animals for 1 h), resulted in more diffuse labeling patterns with no obvious concentration of ^{35}S. This finding could be caused either by slow oxidation of sulfide occurring throughout the gills or by the translocation of ^{35}S-labeled sulfur compounds from the initial sites of oxidation to other regions of the gill cells.

Figures 4 and 5, light and electron micrographs of sections through gill tissue, respectively, show the sulfide oxidizing bodies, the bacteria, and the locations in the gill where the bacteria and sulfide oxidizing bodies were found. Sulfide oxidizing bodies are found throughout the gill. They are most concentrated in Region 2 in Figure 4. Sulfide oxidizing bodies were identified by their size and location within the gill.

Sulfur in S. reidi *gill tissue*

A further demonstration of the distribution of sulfur compounds in the gills of *S. reidi* was provided by X-ray microanalysis of thin sections. This method detects all chemical forms of sulfur present. The X-ray microanalyses revealed high concentrations of sulfur in the sulfide oxidizing bodies, concentrations that were much higher than those in the surrounding tissue (data not shown). This finding provides further support for the role of sulfide oxidizing bodies in sulfide metabolism. Sulfur was not found in or near the bacteria at levels higher than the background levels in the surrounding cytoplasm lacking sulfide-oxidizing bodies. However, TEM examination of gill sections revealed structures in the bacteria similar in appearance to the sulfur globules found within the bacteria of another symbiont-containing clam *Lucina floridana* (Vetter, 1985).

Sulfide oxidation activity in S. reidi *foot tissue*

Figure 6 shows the results of staining transverse sections of *S. reidi* foot tissue for sulfide oxidizing activity. Only the cells comprising the surface layer of the foot stained positively with the benzyl viologen stain. No staining occurred when either sulfide or BV were applied alone. Unlike the staining observed in the gill tissue, the staining in the foot tissue is not restricted to particular observable structures; activity seems to be distributed throughout the cells. Examination of foot tissue of *S. reidi* by transmission electron microscopy failed to reveal bacteria.

DISCUSSION

A major objective of this study was to determine where the initial steps of sulfide oxidation occur in this symbiosis. The data we have presented suggest strongly that in gill tissue of *S. reidi*, the first steps in sulfide oxidation occur not in the bacterial symbionts, but in intracellular organelles we have termed sulfide oxidizing bodies. These organelles were the only sites within the gills to stain positively for sulfide-oxidizing activity, and they were the major site of sulfur compound accumulation as

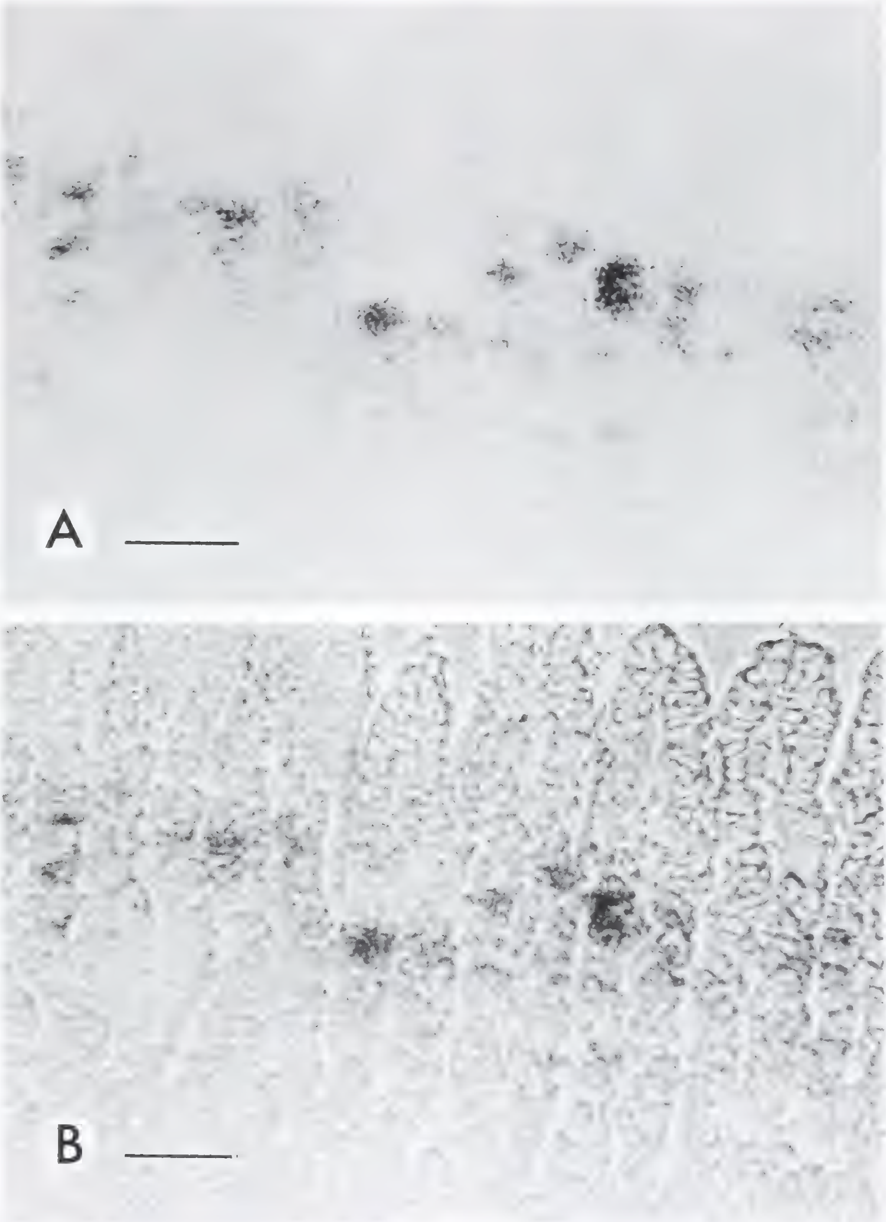

FIGURE 3. Results of a 1 min incubation of *Solemya reidi* gill in ^{35}S-sulfide. The tissue was then sectioned, exposed, and developed as described in methods. Scale bar = 50 μm. (A) Bright-field image of unstained section (same field of view as Fig. 3B). Tissue topography is barely visible, dark spots are silver grains representing sites of label accumulation. (B) Phase-contrast image of unstained section (same field of view as Fig. 3A). Tissue topography is visible, dark spots are silver grains.

observed with autoradiography and X-ray microanalysis. In addition, on density gradients the sulfide oxidizing bodies and sulfide oxidizing ability copurified; the bacterial fraction in the gradient was low in sulfide oxidizing activity.

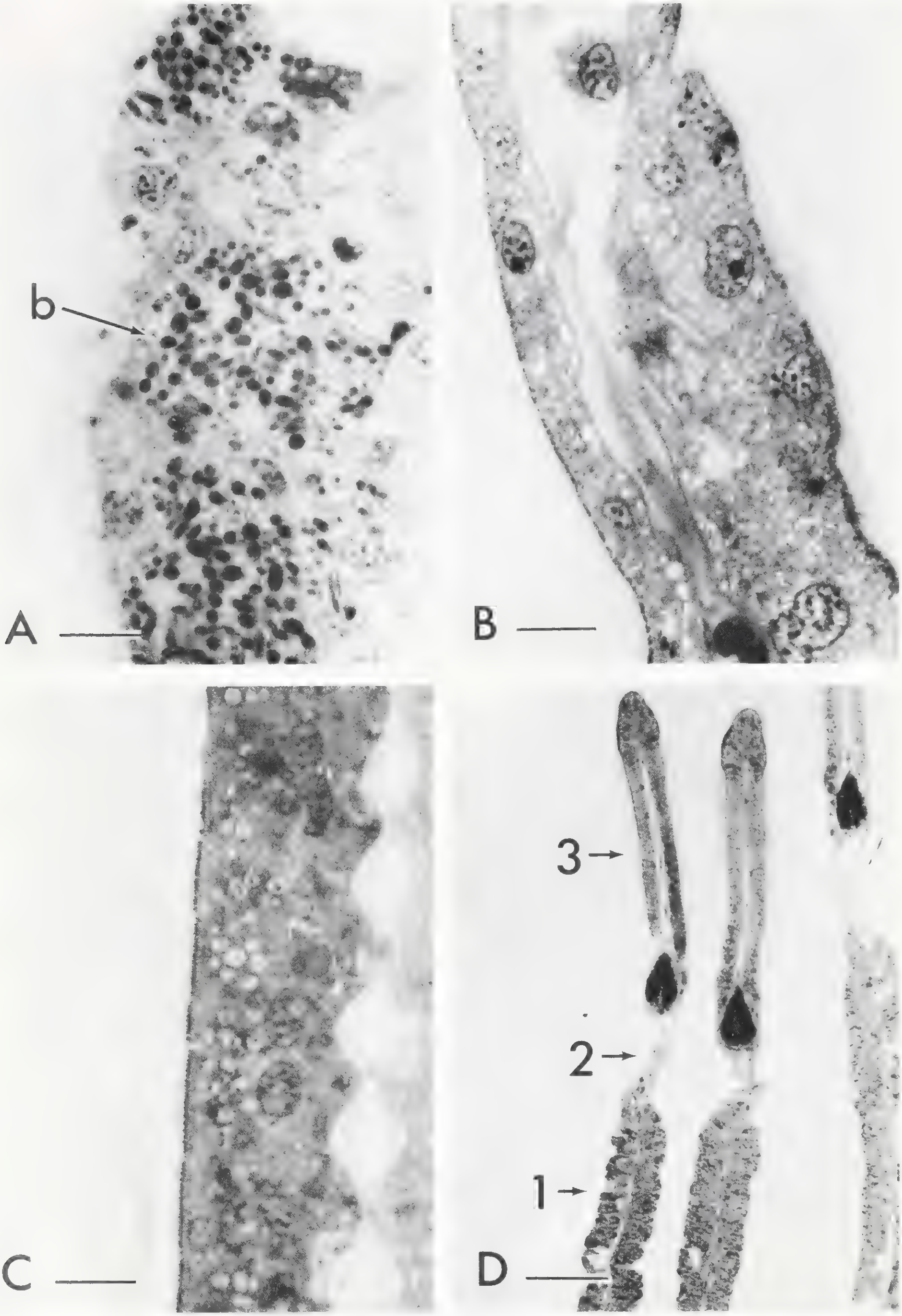

FIGURE 4. Light micrographs of 1 μm transverse section through *Solemya reidi* gill. The section was stained with methylene blue. A—Detail of bacteria-containing portion of gill, b indicates darkly stained bacteria, scale bar = 10 μm. B and C—Detail of portions of the gill which lack bacteria, scale bar = 10 μm. D—Low magnification view of three lamellae of the gill, 1 indicates the position of the types of cells shown in A, 2 indicates the position of the types of cells shown in B, and 3 indicates the position of the types of cells shown in C; the obvious dark object between 2 and 3 is the chitinous skeletal element found at the border between the filaments (3) and the subfilamental region (1 and 2); scale bar = 100 μm.

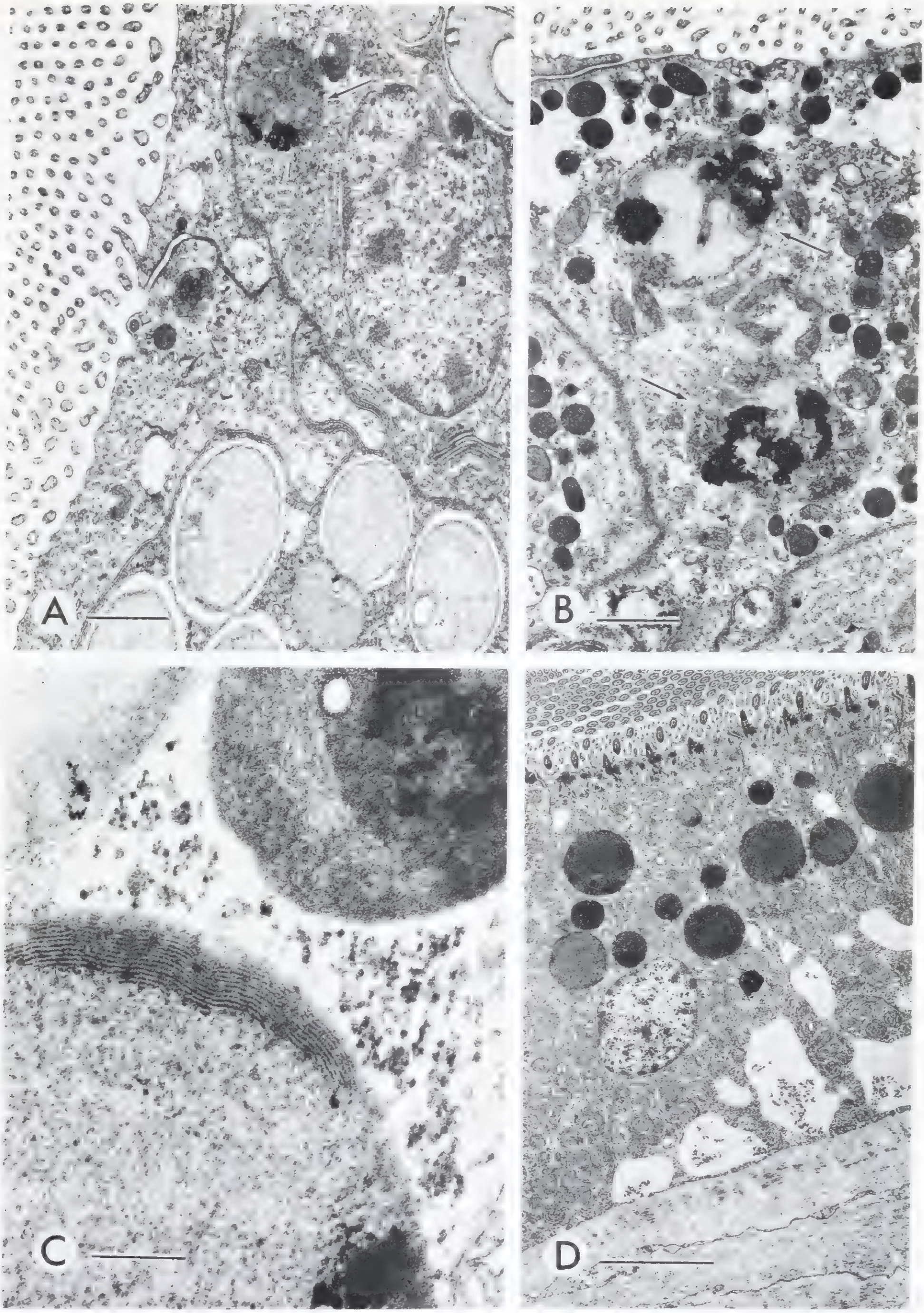

FIGURE 5. Electron micrographs of transverse sections through *Solemya reidi* gill. A—Bacteria-containing portion of gill (1 in Fig. 4D), bacteria are obvious at the bottom and top right, arrow indicates sulfide oxidizing body, the surface of the gill is on the left; scale bar = 1 μm. Sulfide oxidizing bodies are sometimes found in the same cells as bacteria. B—Bacteria-lacking portion of gill (2 in Fig. 4D), arrows indicate sulfide oxidizing bodies, surface of the gill is at the top; scale bar = 1 μm. C—Detail of bacteria-lacking filament of the gill (3 in Fig. 4D), part of three sulfide oxidizing bodies are shown; scale bar = 0.2 μm. D—Lower magnification view of bacteria-lacking filament of the gill, sulfide oxidizing bodies are the obvious darkly stained objects, many mitochondria are also present, the ciliated surface of the filament is at the top; scale bar = 10 μm.

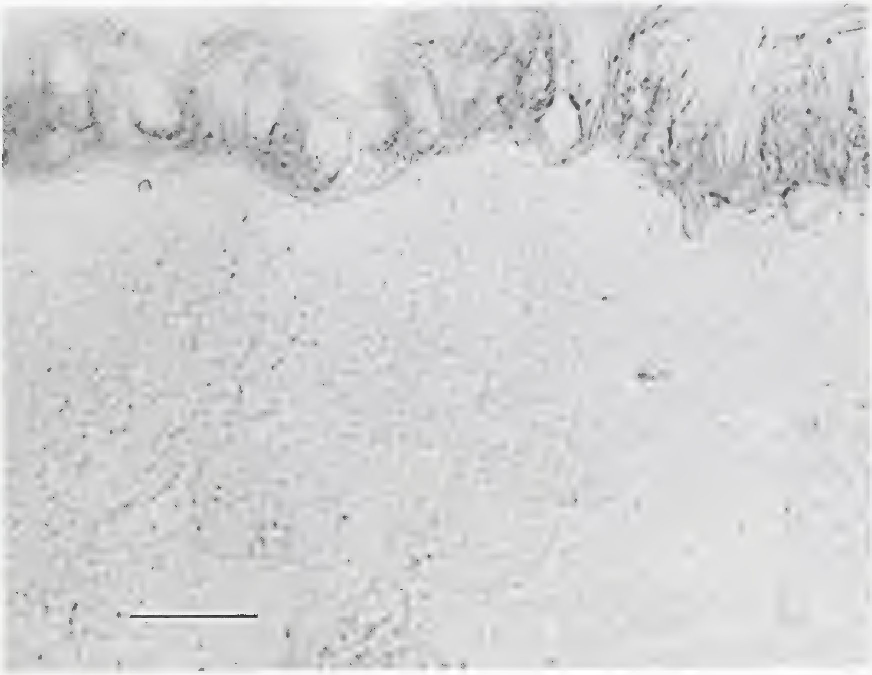

FIGURE 6. Representative sulfide oxidation activity stain of 6 μm transverse section through *Solemya reidi* foot. Scale bar = 10 μm. The dark region is positively stained epithelial cells comprising the surface of the foot. Light regions in the surface cells are unstained nuclei.

The finding that the sulfide oxidizing bodies are the apparent site of the initial oxidation of sulfide has several implications for the overall scheme of sulfur metabolism in this symbiosis. First, the rapid oxidation of sulfide to other sulfur compounds in the sulfide oxidizing bodies may serve as a means for protecting aerobic respiration of the cells from sulfide. We hypothesize that the sulfide oxidizing bodies convert sulfide that diffuses into the gill cells to less toxic or non-toxic forms of sulfur. These partially oxidized forms of sulfur are then provided to the bacteria, where the energy-yielding reactions of chemoautolithotrophic metabolism that form ATP and reducing power are thought to occur (Felbeck, 1983).

A second implication of our observations, however, is that at least some of the energy released in the oxidation of sulfide could be used directly by the animal portion of the symbiosis. We do not know what reactions occur in the sulfide oxidizing bodies, but the initial conversion of sulfide to elemental sulfur, for example, is marked by a negative free energy change of 210 kjoules/mol (Jannasch, 1984). This large negative free energy change could, in theory, provide the driving force for ATP synthesis or pyridine nucleotide reduction in the sulfide oxidizing bodies. Although the animal portion of the symbiosis appears to lack the enzymes for net CO_2 fixation (the Calvin-Benson cycle), the animals may have a substantial capacity for using energy obtained from sulfide oxidation, much as some sulfide oxidizing bacteria are capable of doing (Jannasch, 1984; Somero, 1984).

In foot tissue, where no bacterial symbionts could be detected, all of the sulfide oxidizing activity detected with the cytochemical benzyl viologen method was localized in the superficial cell layer. We hypothesize that in foot tissue, and in other tissues lacking symbionts, a "peripheral defense" strategy for prevention of sulfide inhibition of aerobic respiration may be used. All sulfide diffusing into the tissue is rapidly and quantitatively oxidized to a non-toxic sulfur compound. The foot tissue presumably requires protection because it contains high levels of cytochrome c oxidase (Hand and Somero, 1983), which was greater than 90% inhibited by 5 μM sulfide (data not shown). It remains to be determined whether the energy released in these oxidations can be used to drive ATP synthesis or to reduce pyridine nucleotides; moreover, the fate of the oxidized sulfur compounds remains to be determined. Powell *et al.* (1979, 1980) described a similar case of body-wall sulfide oxidation as a mechanism of sulfide resistance in marine invertebrates lacking symbionts and inhabiting sulfide-rich zones.

The systems responsible for the initial steps in sulfide oxidation in foot and gill of *S. reidi* may be specific "sulfide oxidase" enzymes. This conjecture is based on the observation that extracts of these two tissues contained sulfide oxidizing systems that displayed Michaelis-Menten kinetics. Other tissues tested, as well as the purified proteins (not sulfide oxidases) we examined, did not display this type of kinetics. The sensitivity of the sulfide oxidizing activity to protease treatment and boiling is a further argument for a protein-based activity. All tissues studied, however, showed some capacity for reducing benzyl viologen when sulfide was present. This finding is not surprising in view of previous observations that sulfide oxidation is accelerated in the presence of proteins and metal ions (Baxter and Van Reen, 1958; Sorbo, 1960; Chen and Morris, 1972; Almgren and Hagstrom, 1974). We propose, however, that in animal-bacterial symbioses from sulfide-rich environments, the flow of sulfide into the cells is so great that specific enzymes, *i.e.*, sulfide oxidases, are needed to allow the organism to cope adequately with the threat posed by sulfide and to exploit effectively the energy in sulfide. Sulfide oxidases have been found in sulfide oxidizing bacteria (Moriarty and Nicholas, 1969), but not purified and characterized. Sulfide oxidation in animal tissues has not been shown to be due to the action of specific "sulfide oxidase" enzymes. Thus, little is known about this class of enzymes.

The present study was an initial examination of where and how sulfide is metabolized in *S. reidi*, and many additional questions must be answered before a comprehensive understanding of this symbiosis is possible. It is necessary to determine if the energy released during sulfide oxidation in the sulfide oxidizing bodies is trapped in a useful form, *e.g.*, as ATP. If the animal compartment of the symbiosis is able to obtain a large amount of energy from sulfide oxidation, then the role of the sulfide oxidizing bacteria in the animal's nutrition may be less than would be true if all of the sulfide oxidation was occurring within the bacteria. The nature of the sulfur compounds "fed" to the bacteria by the animal remains to be determined as well. For the symbiont containing clam *Lucina floridana*, Vetter (1985) demonstrated that large amounts of elemental sulfur are present within the bacteria in the gills of freshly collected specimens, and that the elemental sulfur levels decrease during lengthy holding of the clams in sulfide-free seawater. He has proposed that elemental sulfur may serve as a form of energy storage which can be exploited when ambient sulfide levels are low. The same strategy may occur in *S. reidi*, where we observed sulfur globules in the bacteria, and a characteristic change in gill color during storage that resembled that found for *Lucina floridana* (authors' unpub. obs.). Elemental sulfur may be the form of sulfur "fed" to the bacteria by the host; it is the most reduced inorganic sulfur compound which is nontoxic to the host.

The existence of sulfide oxidation activity in the tissues (with and without symbionts) of sulfide-habitat animals suggests that such activity may be vital to the animals. In the symbiont-containing tissues, insufficient detoxification of sulfide might result if sulfide diffused through the animal cytoplasm to the bacteria and was oxidized only by the bacteria. The high levels of sulfide oxidation activity found in the symbiont-containing gills relative to the symbiont-free foot may reflect high demands for reduced sulfur compounds for bacterial metabolism and the requirement that animal cells be protected from sulfide.

In conclusion, the protection of the cytochrome c oxidase systems in mitochondria of gill and foot cells of *S. reidi* appears to be affected by sulfide oxidase enzyme systems which can rapidly oxidize sulfide entering the cells into non-toxic forms of sulfur. These sulfide oxidase systems may as well serve important roles in the generation of ATP and reducing power in the animal cells, a conjecture we now are testing.

Acknowledgments

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REPRODUCTION OF *ANOPLODIUM HYMANAE,* A TURBELLARIAN FLATWORM (NEORHABDOCOELA, UMAGILLIDAE) INHABITING THE COELOM OF SEA CUCUMBERS; PRODUCTION OF EGG CAPSULES, AND ESCAPE OF INFECTIVE STAGES *WITHOUT* EVISCERATION OF THE HOST

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ABSTRACT

Anoplodium hymanae, a member of the turbellarian family Umagillidae, parasitizes the holothuroid *Stichopus californicus* along the northeastern Pacific coast. As in several other species of *Anoplodium,* egg capsules are released into the perivisceral coelom of the host. The egg capsules of *A. hymanae* become ensheathed by host coelomocytes and are then accumulated in masses that are usually about 1 mm in diameter. A single mass from a host that is moderately infested by *Anoplodium* may contain up to several hundred egg capsules. The masses pass out of the host on a daily basis, presumably through previously undescribed ducts that connect the coelom to the lumen of the posterior end of the rectum. Some masses of coelomocytes with egg capsules may be released if the host eviscerates but, contrary to previous hypotheses, evisceration is not required for completion of the life cycle.

Anoplodium hymanae continues to grow after attaining reproductive maturity. The size of the egg capsules, and thus of the larvae, varies with the size of the parent worm.

INTRODUCTION

The turbellarian family Umagillidae constitutes one of the principal groups of metazoan endoparasites of echinoderms and sipunculans (Hyman, 1955, 1959; Barel and Kramers, 1977). The family belongs to the suborder Dalyellioidea within the order Neorhabdocoelida (Crezée, 1982)—the suborder of Turbellaria that is often hypothesized to have the closest common ancestry with the Monogenea, Digenea, and Cestoda (Bresslau and Reisinger, 1928; Karling, 1970; Brooks *et al.,* 1985; Ehlers, in press).

Approximately 50 species of umagillid flatworms have been described (Cannon, 1982; Komschlies and Vande Vusse, 1980a, b; Shinn, 1983a; Westervelt, 1981; and references therein; see also Kawakatsu, 1983), but the life history has been elucidated for only one species (Shinn, 1983b). That worm, *Syndisyrinx franciscanus,* inhabits the intestine of certain sea urchins (Lehman, 1946). Egg capsules of *S. franciscanus* are released into the intestine of the host and pass out of the host with the feces. Embryogenesis occurs in the sea. Fully developed embryos remain in the egg capsules in a dormant state until the capsules are ingested by a sea urchin. The embryos are induced to hatch by some component of the sea urchin intestinal fluids (Shinn, 1983b).

Eleven species of umagillid flatworms are reported to inhabit the perivisceral coelom of aspidochirote holothuroids. Nine of these belong to the genus *Anoplodium* and, as

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far as is known, live exclusively in the coelom. The other species, *Macrogynium ovalis* and *Umagilla forskalensis*, are reported to live in both the coelom and intestine (Snyder, 1980; Cannon, 1982; and references therein). The reproductive biology of coelom-inhabiting umagillids must be more complex than that of intestine-inhabiting species because mechanisms must exist for infective stages of the former to escape from the body cavity of one host and enter the body cavity of a new host.

Several species of umagillid flatworms that inhabit the coelom of holothuroids deposit their egg capsules directly into the coelom (*Anoplodium parasita:* Schneider, 1858; Changeux, 1961; *A. stichopi:* Jespersen and Lützen, 1971; and an unspecified species: Arvy, 1957). The capsules accumulate in masses of host coelomocytes, commonly known as brown bodies. Schneider (1858) observed that coelomic egg capsules of *A. parasita* sometimes contain fully developed embryos but that coelomic capsules are never hatched. He inferred that the capsules pass out of the host before hatching. Changeux (1961) made similar observations on this species and suggested that the egg capsules are cast out when the hosts, *Holothuria tubulosa, H. polii,* and *H. stellata,* eviscerate (auto-expel the intestine and respiratory trees).

Jespersen and Lützen (1971) discovered that brown bodies containing egg capsules of the umagillid *Anoplodium stichopi* are, indeed, cast out of the coelom when its host, the holothuroid *Stichopus tremulus,* eviscerates. There is also evidence that spontaneous evisceration is involved in the release of infective stages of some species of the bizarre entoconchid gastropods and gregarine protozoans that inhabit holothuroids (Tikasingh, 1962; Lützen, 1979). These parasites develop in association with the host's viscera but typically protrude into the perivisceral coelom. As they mature, some species break their connection to the host organs and come to lie free in the coelom.

Many species of holothuroids have been observed to eviscerate in the laboratory, and are thought to eviscerate at least occasionally in the field (Bakus, 1973). In addition to *Stichopus tremulus,* these include *Holothuria forskali* (= *H. nigra*) which harbors the umagillid *Umagilla forskalensis* (Minchin, 1892; Westblad, 1953; Cannon, 1982), *H. tubulosa,* which harbors *Anoplodium parasita* (Schneider, 1858; Changeux, 1961), and *Stichopus californicus,* which harbors *A. hymanae* (Swan, 1961; Shinn, 1983a), as well as many species that are not reported to be parasitized by coelomic umagillids. For the species of *Holothuria,* evisceration is apparently only sporadic. In contrast, *Stichopus californicus* and at least a portion of some populations of *S. tremulus* have been thought to eviscerate spontaneously every autumn (Swan, 1961; Jespersen and Lützen, 1971, respectively). Lützen (1979) suggested that seasonal evisceration provides a dependable means of escape for infective stages of parasites infecting the coelom of at least some holothuroids.

Contrary to what one would expect if seasonal evisceration was the only, or most important, means of escape of infective stages from the coelom of *Stichopus californicus* or *S. tremulus,* maturation of their coelomic parasites, and formation of egg capsules or other infective stages by the parasites are not seasonally restricted (Lützen, 1979). Recently, Fankboner *et al.* (1981) reported that the viscera of *S. californicus* undergo a seasonal resorption and regeneration rather than evisceration. Similarly, *S. japonicus,* which harbors the umagillid *A. mediale,* resorbs the gut seasonally and is not known to eviscerate annually (Ozaki, 1932; Tanaka, 1958). While there is clearly a need for more information about the natural occurrence of evisceration, these observations suggest that means other than spontaneous evisceration exist for the passage of infective stages of coelom-inhabiting parasites from the host.

Kincaid (1964) hypothesized that entoconchid gastropods can induce evisceration of the host at times that are appropriate for release of infective stages. Lützen (1979) discounted this hypothesis because the host that Kincaid was studying, *Stichopus* *californicus*, as well as *S. tremulus* which also harbors entoconchids, are virtually never found with regenerating viscera except during what has been thought to be the fall evisceration period. It has been suggested that infective stages are liberated when the hosts die, but Lützen (1968, 1979) mentioned that holothuroids are probably relatively long-lived and that infective stages are more likely to be eliminated during even occasional eviscerations of the hosts. Massin *et al.* (1978) suggested that gregarine protozoans that inhabit the deep sea holothuroid *Psychropotes longicauda* might use intermediate hosts, such as eulimid gastropods that suck the coelomic fluid out of holothuroids, to help them escape the coelom. There is, however, no evidence to support this interesting possibility. None of these hypotheses are as attractive as that of spontaneous evisceration, but there have been no attempts other than Lützen's (1979) to solve this problem in detail.

This paper describes certain aspects of the life history of *Anoplodium hymanae* Shinn. This umagillid inhabits the coelom of the aspidochirote holothuroid *Stichopus californicus* in the northeastern Pacific. The morphology of egg capsules, rate of production of egg capsules, duration of embryogenesis, and the importance of evisceration for release of infective stages are analyzed. In addition, I describe experiments which reveal that infective stages escape the coelom on a regular basis in the absence of evisceration of the host. A new morphological feature of stichopodid holothuroids, by which escape of infective stages may be accomplished, is described. The mechanism of invasion of new hosts, the morphology of larvae, and seasonal variations in the intensity of infestation are described in another paper (Shinn, 1985). These results have been summarized in an abstract (Shinn, 1983c).

MATERIALS AND METHODS

Collection and dissection of hosts

Specimens of *Stichopus californicus* were collected by divers near Colin's Cove, San Juan Island, Washington (48°33′N, 123°00′W, 2–15 m deep). The sea cucumbers were all more than 20 cm long. They were kept in running sea water aquaria at the Friday Harbor Laboratories (FHL). Those used for censusing were dissected within a few days of collection.

Sea cucumbers were sliced open with a razor blade and the coelomic fluid was collected in a dish. The viscera and body wall were submerged in sea water in a second dish. The dishes were placed on black cloth so that the white worms would be more visible. Worms were transferred via a Pasteur pipette to a separate container of filtered sea water. Brown bodies (*i.e.*, the masses of coelomocytes that contain egg capsules) were collected from the viscera, the inner side of the hosts' body wall, and the bottom of the dishes. After the coelomocytes settled out of suspension in the dish of coelomic fluid (about 1 h), the fluid was decanted and the bottom of the dish was examined with a dissecting microscope for additional small worms and egg capsules.

Maintenance of animals and egg capsules

Flatworms and egg capsules were kept at low densities in filtered sea water. Short, wide-mouth jars were used as containers; they could be capped loosely in order to prevent evaporation. A large air space was left in the jars. The sea water was usually changed every two days. The jars, and 20 liter aquaria in which sea cucumbers were kept during experiments, were partially submerged in continuously flowing water in sea tables. The temperature ranged from 8–12°C depending on the time of year. Unless specified, the light regimen was not controlled; it consisted of alternating periods of subdued light (from windows) and dark.

To determine the rate of production of egg capsules, adult worms were dissected from sea cucumbers and kept in the dark. The worms appeared healthy for 3–5 days under these conditions. The numbers of egg capsules deposited by the worms during the first 48 h were used to calculate rates of capsule production. Capsules produced and released by these worms were kept in separate containers of filtered sea water. Embryogenesis was followed by periodically examining the intact capsules with a compound microscope.

Effects of evisceration

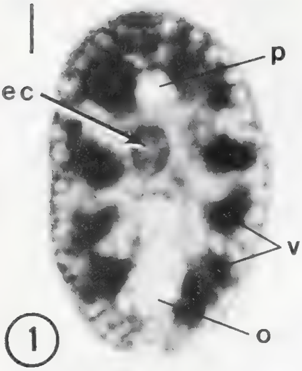
When recently collected holothuroids eviscerated in aquaria, expelled worms and brown bodies with egg capsules were collected and counted. The eviscerated holothuroids were isolated in separate aquaria for one day and then dissected. Worms and egg capsules remaining in the coelom were counted. Holothuroids were judged to have eviscerated in the field when the following criteria were met: (1) they did not eviscerate after collection, and (2) the digestive tract consisted only of the open-end rectum and a stub of the intestine attached to the aquapharyngeal bulb. Specimens that had a regenerating intestine were not categorized as having eviscerated because of the possibility of confusing specimens that had eviscerated with those that had resorbed the digestive tract (see Fankboner *et al.*, 1981).

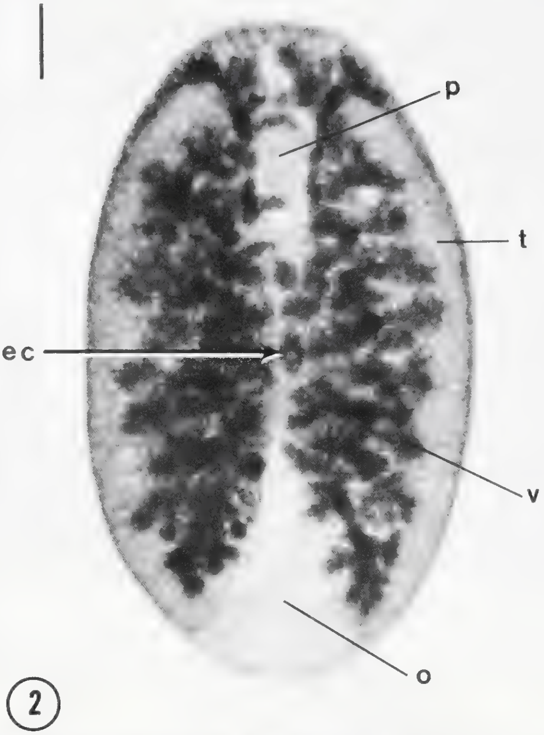
Release of infective stages from the host

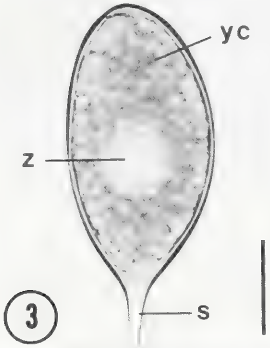
To determine if egg capsules or worms are voided from hosts by mechanisms other than evisceration, specimens of *Stichopus californicus* were put into individual 20 liter aquaria that had been fitted with a 0.5 cm mesh screen four cm above the bottom. This arrangement allowed objects coming out of the holothuroids to settle through the mesh, and thus prevented ingestion of these objects by the sea cucumbers. Sea water was allowed to trickle into the aquaria from above. The sea cucumbers were not fed. Every two days the water in the aquaria was filtered through a 143 μm Nitex screen and examined for brown bodies, egg capsules, and worms. At the end of the experiments (7–20 days), the animals were dissected. The worms and egg capsules in the coelom were counted. This procedure was repeated at different times of year using a total of eight *S. californicus.*

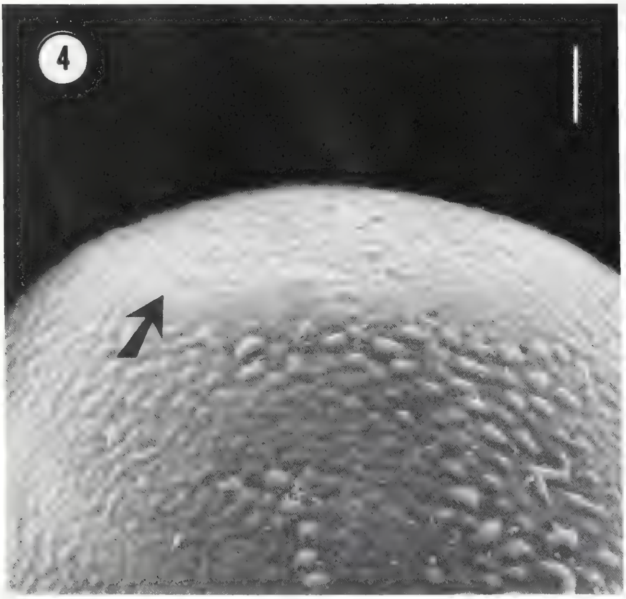
To locate coelomic ducts through which egg capsules of *Anoplodium hymanae* might escape the host, the perivisceral coelom of each of several large *Stichopus californicus* was injected with 10 ml of a 1% solution of methylene blue in sea water (technique of Anderson, 1966). The animals were held with the right side uppermost so that gravity would pull the internal organs away from the site of injection; this was located midway along the right side of the body. At various times over the next two days, the injected specimens were anesthetized in a 1:1 solution of 7.5% $MgCl_2$:sea water. They were then submerged in a shallow dish, and examined with the aid of a dissecting microscope. Pressure was applied to the body in order to force the dye out of any openings that might exist in the body wall.

To determine if egg capsules are transported out of the host by migration of egg capsule-laden masses of host coelomocytes through the body wall, or wall of the intestine, rectum, or respiratory trees, these parts of highly infested sea cucumbers were examined with the dissecting microscope. Dark masses of cells resembling brown bodies were teased apart with insect pins, or were excised, mounted on a slide, and examined with a compound microscope for the presence of egg capsules. Large pieces of the digestive tract and respiratory trees were also compressed between two slides, then examined with a compound microscope. In addition, the body wall of two *Stichopus californicus* that each contained more than 100 *Anoplodium hymanae* were









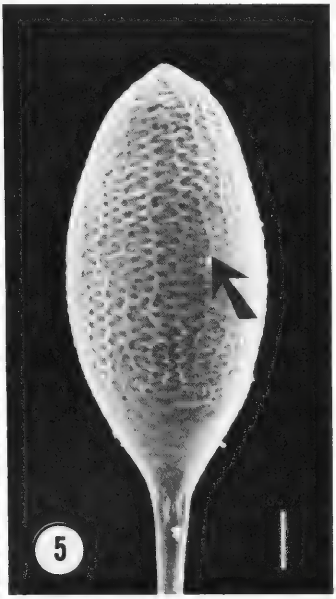


FIGURE 1. Small reproductive specimen (live) of *Anoplodium hymanae;* dorsal view. Photomicrograph; ec, egg capsule; o, ovary; p, pharynx; v, vitellaria. Scale = 100 μm.

FIGURE 2. Large reproductive specimen (live) of *A. hymanae;* ventral view. The egg capsule (ec) is larger in absolute size but much smaller relative to the size of the parent than the egg capsule of the specimen in Figure 1. Photomicrograph; o, ovary; p, pharynx; t, testis; v, vitellaria. Scale = 250 μm.

digested in papain (Adolph's Meat Tenderizer) and the digestate was examined for egg capsules. Before treatment, the body walls were washed thoroughly with fresh water. The body walls were then sliced into pieces and placed in a large beaker along with about 50 ml of fresh water and approximately 100 gm of the enzyme preparation. Digestion was carried out at room temperature for 48 h. Control egg capsules that had been dissected out of sea cucumbers were treated in a papain solution of the same concentration for the same duration.

Electron microscopy

For scanning electron microscopy, egg capsules deposited by worms kept in sea water, and brown bodies collected from hosts, were fixed in phosphate-buffered OsO_4 (Cloney and Florey, 1968). The capsules were punctured with a razor blade soon after immersion in the primary fixative; if not punctured, the capsules collapsed during dehydration. The capsules were dehydrated in an ethanol series, transferred to 2,2-dimethoxypropane, critical point dried from CO_2, coated with Au/Pd, and examined with a JEOL JSM-35 scanning electron microscope (SEM).

RESULTS

Anoplodium hymanae is hermaphroditic. It becomes reproductive when it reaches a length of about 0.5 mm, and it grows to a maximum length of about 2.5 mm (Figs. 1, 2). Mature worms never contain more than a single egg capsule; these capsules always contain very young embryos. Thus, egg capsules are produced and released into the coelom one at a time, in continuous succession.

Morphology of egg capsules

Egg capsules of *Anoplodium hymanae* consist of an oblong bulb that is drawn out at one end into a hollow filamentous stalk. Newly produced capsules contain a single zygote (rarely 2–3 zygotes) and several dozen yolk cells (Fig. 3). The egg capsules measure 75–200 μm long by 45–97 μm wide. They vary in size with the size of the parent. Zygotes and yolk cells are of fairly constant dimensions (36–40 μm diam. and 15–18 μm diam., respectively). Capsules produced by large worms contain many more yolk cells than capsules produced by small worms. Small capsules are smooth or decorated by small verrucae (Fig. 4); capsules longer than about 160 μm have an elaborate pattern or ridges on the outside (Fig. 5). The anterior end of the bulb bears an opercular suture (Figs. 4, 10). Both the bulb and stalk are formed from secretions of the yolk cells. In some other species of umagillids, the "stalk" is a separate structure formed of secretions of the so-called cement glands (*e.g., Syndesmis echinorum,* Meixner, 1923; *Syndisyrinx franciscanus,* Shinn, 1983b). *Anoplodium hymanae* lacks cement glands (Shinn, 1983a).

FIGURE 3. Egg capsule of *A. hymanae* released *in vitro.* The capsule has a bulbous part that contains a zygote (z) and numerous yolk cells (yc); the stalk (s) of the capsule is continuous with the wall of the bulbous part. Scale = 50 μm.

FIGURE 4. Scanning electron micrograph of distal ¼ of medium-sized egg capsule of *A. hymanae;* capsules of this size are covered by small bumps. The position of the opercular suture is indicated by an arrow. Scale = 10 μm.

FIGURE 5. SEM of bulb of large egg capsule of *A. hymanae.* The bulbous part of the capsule is decorated by a network of longitudinal ridges with interdigitating side branches (arrow). Scale = 25 μm.

As noted in the original description of *Anoplodium hymanae,* specimens in a single host are often of two conspicuously different sizes (Shinn, 1983a). Egg capsules recovered from these hosts are commonly of two distinct sizes.

Rate of egg capsule production and duration of embryogenesis

Small reproductive specimens of *Anoplodium hymanae* that were kept in sea water produced and released egg capsules at a rate of about one per day (Table I). Large worms produced an average of about nine normal capsules a day, though some specimens produced considerably more (Table I). All large worms produced some abnormal egg capsules. The latter were usually narrower than normal capsules and were incomplete at the anterior end so that they would not hold eggs or yolk cells. Abnormal capsules are sometimes found in the coelom of *Stichopus californicus* but usually not in so high a proportion to normal capsules as was produced *in vitro.* The abortive capsules produced *in vitro* might have been normal if the worms had been in their hosts. Including the abnormal capsules, large worms produced an average of about 13 capsules per day.

Embryos in egg capsules that were deposited *in vitro* completed development in 30–35 days (see *Morphology of larvae* section in Shinn, 1985). Fully developed larvae were typically quiescent but were capable of ciliary and muscular movement. The embryos survived in the capsules for over ten months after development was completed. The embryos eventually died without hatching.

Status of egg capsules in the coelom

Egg capsules of *Anoplodium hymanae* occur either singly in the perivisceral coelomic fluid of the host or, more commonly, grouped within spherical masses of host coelomocytes (Figs. 6, 7). The masses of coelomic cells vary from a fraction of a mm to over one mm in diameter, and contain 25–300 egg capsules in sea cucumbers that are moderately infested. Most of the coelomocytes composing the masses are irregular in shape and contain numerous yellow-brown inclusions that give the masses an orange to brown color. The masses of coelomocytes are referred to as "brown bodies" (Hyman, 1955; Hetzel, 1965). Some brown bodies may be free in the coelom; they come out when the host is sliced open. Others adhere to the peritoneum lining the body wall. Brown bodies with egg capsules are particularly abundant among the suspensors that connect the rectum to the body wall.

TABLE I

Production of egg capsules by Anoplodium hymanae *kept in sea water*

	Small worms (0.7–1.0 mm long)		Large worms (2.0+ mm long)	
	Mean (n = 16 worms)	Range for individual worms	Mean (n = 16 worms)	Range for individual worms
Number of normal capsules released in 48 h	1.6	0–3	18.6	12–32
Number of normal plus abnormal capsules released in 48 h	1.6	0–3	26.6	16–38

Most of the egg capsules that occur singly in the coelom contain young embryos that have not begun to incorporate yolk cells. Egg capsules within brown bodies contain embryos in various stages of development, including many that have completed development (Figs. 8, 9). In a sample of 1500 capsules recovered from the coelomic brown bodies of six hosts, 1443 (96%) contained live embryos, 28 (2%) contained dead and disintegrating embryos (Fig. 10), and 25 (2%) had the operculum dislodged and lacked an embryo. Embryos in 4 (0.3%) capsules were in the process of hatching. Most of the capsules containing dead embryos and all of the capsules that were open or hatching were of the size produced by small worms. A cursory examination of egg capsules in brown bodies of other hosts (more than 70 sea cucumbers) revealed that no hosts contained more than a dozen opened capsules.

Host reaction to egg capsules

Some of the single egg capsules in the coelom are naked but most single capsules and all capsules in brown bodies are individually ensheathed by one or more layers of flattened coelomocytes (Fig. 11). The ensheathing cells usually lack the yellow-brown inclusions that are characteristic of other coelomocytes in brown bodies. In general, the ensheathed capsules remain intact and the embryos continue to develop. Coelomocytes were present, however, inside the bulb of the few open and empty capsules that were present in brown bodies.

The individual ensheathment of egg capsules of *Anoplodium hymanae* appears to constitute a specific host reaction to the capsules, but formation of brown bodies does not occur solely in response to the presence of egg capsules of *A. hymanae.* Brown bodies are present in uninfested specimens of *Stichopus californicus,* and some brown bodies in infested specimens are devoid of egg capsules. Brown bodies in the polian vesicles of parasitized and unparasitized sea cucumbers do not contain egg capsules. Some brown bodies consist entirely of host coelomocytes. Others contain calcareous ossicles of host origin, gamontocysts of gregarine protozoans, or occasionally, diatom frustules or dead copepods, in addition to, or instead of, egg capsules of *A. hymanae.*

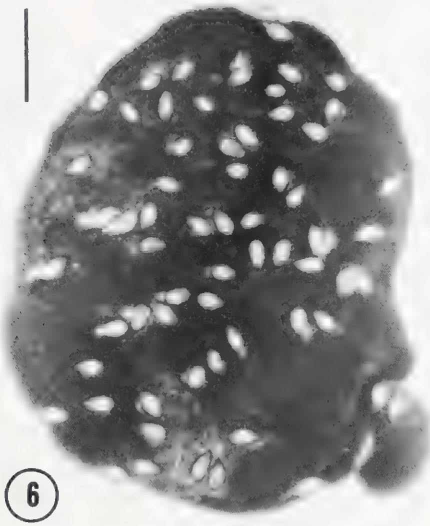
Effects of evisceration

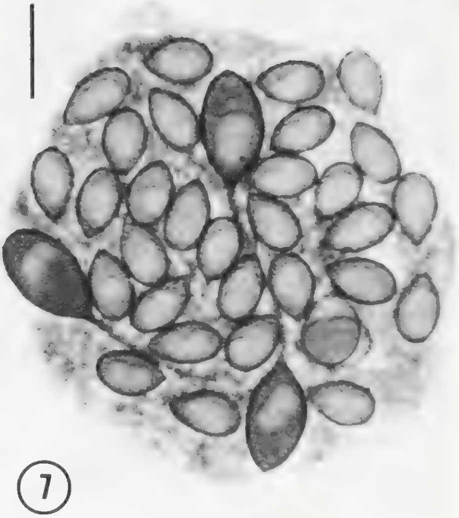
Two specimens of *Stichopus californicus* were observed eviscerating in aquaria on the day after they had been collected (31 Aug. 1982). Specimens of *Anoplodium hymanae,* as well as brown bodies with egg capsules, were expelled along with the hosts' intestine and respiratory trees (Table II). In both cases, evisceration was far from effective in eliminating all egg capsules or worms from the host. Of the 75+ sea cucumbers dissected during this study (see Shinn, 1985), only one had eviscerated in the field before it was collected (18 Nov. 1981); it contained 7 worms and 98 egg capsules.

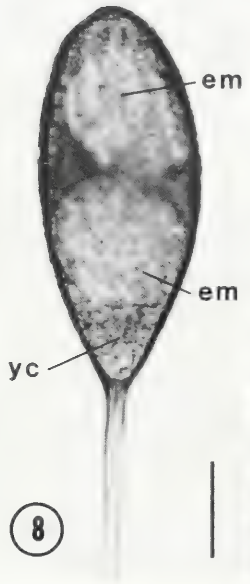
Release of infective stages without evisceration

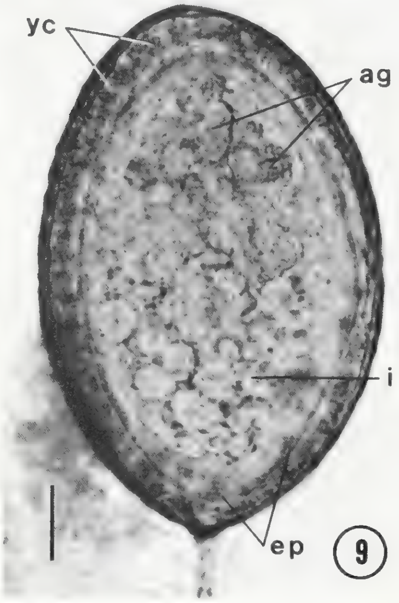
Egg capsules of *Anoplodium hymanae* were expelled in large numbers at frequent intervals from all of eight *Stichopus californicus* that were kept in individual screen-bottomed aquaria (Table III). All of the sea cucumbers had fully differentiated viscera when dissected at the end of the experiment. Nearly all of the expelled egg capsules were enclosed within brown bodies.

The brown bodies passed from the hosts were about 1 mm in diameter. They contained between 8 and 385 (mean 81) capsules. The average number of capsules released per day from individual hosts varied from 6 to 214. Egg capsules were released from four *Stichopus californicus* that contained no reproductive worms (Table III).









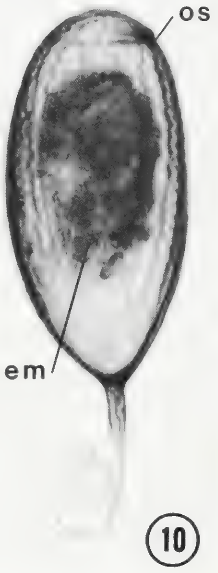


FIGURE 6. Mass of coelomocytes (*i.e.*, a "brown body") recovered from perivisceral coelom of *Stichopus californicus*, containing at least 65 egg capsules of *Anoplodium hymanae;* the volume of host cells filling the areas between egg capsules is large; the egg capsules are all large in size; specimen flattened considerably. Scale = 500 μm.

FIGURE 7. Brown body recovered from the coelom of *S. californicus* containing egg capsules of *A. hymanae* which are of two distinctly different sizes; the large capsules are similar in size to the capsules in the brown body of Figure 6; the volume of host cells filling the areas between egg capsules is small. Scale = 150 μm.

FIGURE 8. Egg capsule of *A. hymanae* removed from a brown body of *S. californicus;* the capsule contains two incompletely developed embryos (em); there are no differentiated organs and the embryos are immobile. The embryos are surrounded by yolk cells (yc) which have not yet been incorporated. This capsule is unusual because most capsules of *A. hymanae* contain only one embryo. Scale = 50 μm.

Similarly, large capsules were released from one host that contained no large worms (Table III). The worms that had deposited the capsules were no longer present in the hosts. Dissections of freshly collected animals have revealed that reproductive worms are also lost in nature (see Shinn, 1985). Rates of capsule release were calculated from data for hosts D and E (Table III) because reproductive worms had not obviously been lost from them. Large capsules were expelled at rates of 31.3 and 9.25 per day per large reproductive worm in the coelom of hosts D and E, respectively; small capsules were expelled at rates of 1.0 and 1.6 per day per small reproductive worm.

In addition to the typical coelomic brown bodies, hundreds of small (most less than 0.2 mm in diameter), dark purple or black masses of host cells that were visibly distinct from the coelomic brown bodies were recovered from the aquaria. These smaller masses never contained egg capsules of *Anoplodium hymanae.* Cells in the smaller masses contained inclusions similar to those in the cells composing coelomic brown bodies. No subadult or mature specimens of *A. hymanae* were recovered outside of the hosts. Fecal strings that did not fall through the mesh were removed when they were noticed and examined with a dissecting microscope. No egg capsules of *A. hymanae* were found in them, although egg capsules of an undescribed species of *Ozametra* that inhabits the intestine of *Stichopus californicus* were frequently found stuck to the mucus that ensheathed the feces.

Pathway of expulsion of brown bodies

The wall of the rectum of *Stichopus californicus* was found to be perforated by a ring of ducts that connect the posterior end of the perivisceral coelom to the lumen of the rectum (Figs. 12, 13). Large specimens typically have about 75 ducts but the number differs between specimens of different size. Thirteen ducts were counted in sections of a 1.5 cm long *S. californicus.* The ducts are located just internal to the anal sphincter and pass straight through the wall of the rectum. They were observed in live, anesthetized specimens by pulling open the anal sphincter and examining the wall of the rectum with the aid of a dissecting microscope. Coelomic brown bodies containing egg capsules and irregularly shaped masses of coelomocytes containing methylene blue that had been injected into the holothuroids could be seen, through the translucent rectum wall, among the suspensors of the rectum. The brown bodies and masses containing methylene blue were easily forced out of the coelom, through the ducts, and out the anus, by applying pressure to the body wall of the sea cucumber. The histological relationship of the ducts to the surrounding tissues will be described in a separate paper (Shinn and Stricker, in prep). No pores or ducts that either directly or indirectly connect the perivisceral coelom to the outside were found in other parts of the body of *S. californicus.*

Neither egg capsules nor large masses of cells identical to coelomic brown bodies were observed within the solid tissues of specimens of *Stichopus californicus.* The body wall, intestine, rectum, and respiratory trees of *S. californicus* typically contain hundreds of small, dark accumulations of cells resembling the small dark masses of cells recovered

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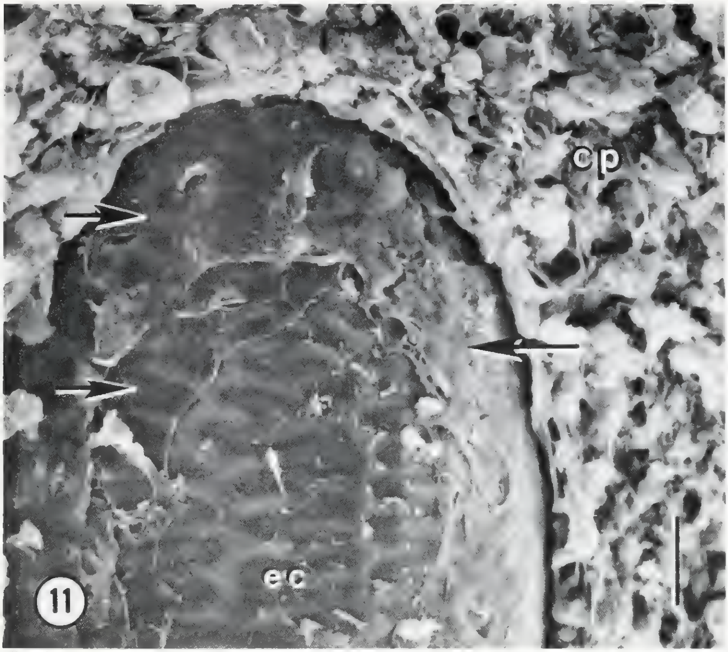
FIGURE 9. Egg capsule of *A. hymanae* containing a fully developed embryo; capsule compressed slightly. The anterior glands (ag), epidermis (e), and intestine (i) are visible; the embryo is capable of ciliary and muscular movement. The egg capsule was removed from a brown body from the perivisceral coelom of *S. californicus.* Scale = 25 μm.

FIGURE 10. Egg capsule of *A. hymanae* containing a dead, disintegrated embryo (em). The opercular suture (os) is conspicuous; the operculum is easily dislodged. The capsule was removed from a brown body in the coelom of *S. californicus.* Scale = 50 μm.



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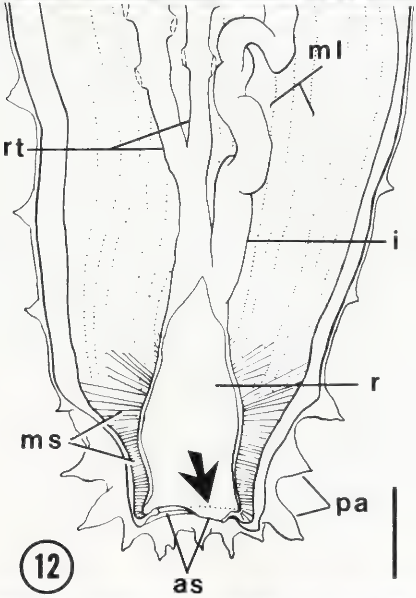

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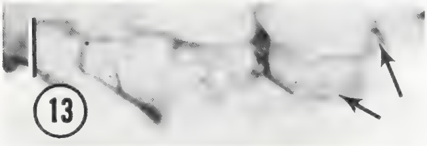

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FIGURE 11. SEM of egg capsule of *Anoplodium hymanae* in a brown body of *Stichopus californicus.* The egg capsule (ec) is ensheathed by flattened host coelomocytes (arrows); the pattern of ridges on the surface of the egg capsule is visible where the sheath of coelomocytes has been removed; cp, coelomocyte packing. Scale = 10 μm.

TABLE II

Numbers of egg capsules and Anoplodium hymanae *expelled at evisceration* versus *numbers remaining in the coelom after evisceration of* Stichopus californicus

	Expelled with viscera		Retained in coelom after evisceration	
Host	Egg capsules	Worms	Egg capsules	Worms
A	221 (8.4%)	1	2405 (91.6%)	19
B	300+	33	300+	10

from the bottom of the aquaria in the capsule-expulsion experiments that were mentioned previously. Of several hundred of these masses that were teased apart or that were examined in squashes of tissues from each of five highly infected sea cucumbers, none contained egg capsules. No egg capsules of *Anoplodium hymanae* were found among the papain-digested remains of the body walls of two *S. californicus* that harbored more than 100 *A. hymanae* each. Control egg capsules were not digested by papain.

Status of embryos in expelled egg capsules

Most capsules expelled by intact hosts contained embryos in an advanced stage of development, or embryos that had completed development (Table IV). Twelve (0.6%) of the 2094 capsules examined were open and empty (Table IV). Although quantitative results were not recorded for capsules expelled during evisceration nearly all of those capsules contained viable embryos in various stages of development.

DISCUSSION

Escape of infective stages

Egg capsules of *Anoplodium hymanae* are deposited into the coelom of the host, *Stichopus californicus,* soon after they are formed. The capsules get ensheathed by small numbers of coelomocytes and, subsequently, accumulated with additional coelomocytes into the large masses that constitute brown bodies. The vast majority of capsules are transported out of the host before hatching, as was hypothesized for *A. parasita* by Schneider (1858). In contrast to previous hypotheses (Changeux, 1961; Jespersen and Lützen, 1971), the capsules are most commonly externalized without evisceration of the host. The existence of permanent ducts between the coelom and the rectal lumen, the accumulation of brown bodies with egg capsules in the vicinity of the ducts, and the fact that brown bodies containing egg capsules can be forced

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FIGURE 12. Line drawing of dissection of posterior end of *S. californicus.* The body wall was sliced open and folded back; the rectum (r) was opened to reveal the openings of the coelomic ducts (arrow) into the lumen of the rectum. The rectum is attached to the body wall by the radiating suspensor muscles (ms); as, anal sphincter; i, intestine; ml, longitudinal muscles of body wall; pa, papillae on outside of body wall; rt, respiratory trees. Scale = 2 cm.

FIGURE 13. Photomicrograph of openings of coelomic ducts (arrows) into the lumen of rectum. Specimen prepared as described for Figure 12, then fixed in formalin to make the tissues opaque. Scale = 0.5 mm.

TABLE III

Numbers of egg capsules of Anoplodium hymanae *released from intact* Stichopus californicus

		No. of egg capsules recovered				No. of reproductive worms in coelom		
		From aquarium			From coelom			
Host	Dates of experiment	Total	Lg.*	Sm.	Total	Total	Lg.	Sm.
A.	3–12 Oct. 81	139 (1)**	nd	nd	76 nd	4	0	4
B.	3–12 Oct. 81	110 (10)	110	0	58 nd	0	0	0
C.	1–8 Dec. 81	90 (10)	1	89	146 nd	24	0	24
D.	6–26 Aug. 82	3327 (19)	3127	200	3888 (44)	15	5	10
E.	6–26 Aug. 82	741 (25)	185	556	300+ (36)	18	1	17
F.	5–25 Sept. 82	107 (4)	nd	nd	472 (40+)	0***	0	0
G.	5–25 Sept. 82	290 (7)	nd	nd	2 (29+)	0***	0	0
H.	5–25 Sept. 82	4071 (41)	nd	nd	79 (55)	0	0	0

* See *Intensity and seasonality of infestation* in Shinn (1985) for detailed explanation of size classes of worms and capsules.

** Numbers in parentheses represent the numbers of brown bodies that contained the capsules.

*** Small, non-reproductive worms were recovered from these hosts

nd = No data.

through the ducts by squeezing anesthetized hosts, strongly suggest that brown bodies with egg capsules are normally expelled through the ducts.

Except for the lesion that forms at the anterior end of the rectum during evisceration, neither permanent nor temporary openings that either directly or indirectly connect the perivisceral coelom to the outside have been described for aspidochirote holothuroids. Coelomic pores have been described for two species of apodan holothuroids (Becher, 1912; Anderson, 1966), however, and pores that form as temporary breaks in the posterior end of the rectum have been described for the molpadonian holothuroid *Caudina chilensis* (Kawamato, 1927; Kitao, 1935). The discovery of coelomic ducts or pores in rather cryptic locations in widely divergent species suggests that such openings may be common in holothuroids. Echinoids may also have cryptic coelomic ducts. Eight species of umagillids are reported to inhabit the intestine and coelom of echinoids (reviewed by Shinn, 1984), and presumably deposit egg capsules into the perivisceral coelom of their hosts. If these reports are valid (see Shinn, 1981), there must be a mechanism by which the egg capsules exit the coelom.

Stichopus californicus has four types of coelomocytes: amoebocytes, morula cells, lymphocytes, and crystal cells (Hetzel, 1963). Hetzel (1965) determined that, of these cell types, only amoebocytes are actively involved in phagocytic activity and the formation of brown bodies in three species of dendrochirote holothuroids and one species

TABLE IV

Developmental stage of embryos in egg capsules of Anoplodium hymanae *released from intact* Stichopus californicus

Date	Host*	Total no. capsules examined	Total no. open capsules	Percent of capsules containing embryos of specific stages**			
				1 Zygote	2 Mid-development	3 Developed	4 Dead
1–8 Dec. 1981	C.	90	0	0	61	20	19
6–26 Aug. 1982	D.	993	3	0	31	69	0
6–26 Aug. 1982	E.	497	9	10	48	42	0
5–24 Sept. 1982	G.	50	0	2	18	78	2
5–24 Sept. 1982	H.	464	0	0	6	94	0
Total		2094	12	3% (n = 11)	37% (n = 146)	55% (n = 215)	5% (n = 18)

* Host symbols correspond to those in Table III.

** Percentage based when possible on a subsample of 100 capsules.

1. Embryos the size of zygotes, epidermal layer not visible (Fig. 3).
2. Medium-sized embryos with a visible epidermal layer but without differentiated internal organs (Fig. 8).
3. Fully differentiated embryos, capable of movement (Fig. 9).
4. Embryos disintegrating (Fig. 10).

of apodan holothuroid. In some echinoids, morula (= spherule) cells as well as amoebocytes are involved in encapsulation of foreign material (reviewed by Smith, 1981). Further study is needed to verify that amoebocytes alone are involved in the ensheathment of the egg capsules of *Anoplodium hymanae* and their accumulation into brown bodies.

Brown bodies are formed by holothuroids, echinoids, and, to a lesser extent, ophiuroids (Smith, 1981; Jangoux, 1982). Structures that have the same name, but that are not homologous, have been reported for asteroids (Johnson and Beeson, 1966; Smith, 1981). Coelomocytes in brown bodies are commonly thought to be degenerate. It has been hypothesized that the yellow-brown inclusions that characterize the cells consist of the metabolic remains of pigments derived from the food (reviewed by Jangoux, 1982), and that brown bodies are formed as a means of sequestering the unwanted materials. The origin, composition, and functions of the inclusions are, however, unknown. Inclusion of foreign materials that have been ensheathed by coelomocytes is presumably an additional function of brown bodies, and is obviously associated with the defense mechanisms of the host.

Many authors have reported that individual coelomocytes, brown bodies, or other masses of coelomocytes containing foreign materials that have been injected into the coelom, can migrate out of echinoderms. In holothuroids this has been reported to involve virtually all tissues of the body (intestine, body wall, respiratory trees, etc.;

Hetzel, 1965), although in members of other classes of echinoderms it may involve only localized parts of the body (reviewed by Endean, 1966). I found no evidence that coelomic brown bodies with egg capsules migrate through the tissues of the body wall or wall of the intestine or respiratory trees of *Stichopus californicus*. My observations were fairly limited and do not disprove the latter possibility, but reveal that expulsion of egg capsules by that process is at least uncommon. The small dark masses of cells in the solid tissues of *S. californicus* and those recovered from the aquarium in my capsule releasing experiments are, by definition, brown bodies. They do not contain egg capsules of *Anoplodium hymanae*, which suggests that they do not originate in the perivisceral coelom. Only brown bodies in the perivisceral coelom contain egg capsules. This observation reflects the fact that *A. hymanae* occurs only in the perivisceral coelom, and it also demonstrates that the host is compartmentalized with regard to the movement of brown bodies that originate in different parts of the body.

Some egg capsules of *Anoplodium hymanae* are released to the sea during evisceration. It is doubtful, however, that the number of capsules expelled from eviscerating holothuroids is increased above the number that would be released in the absence of evisceration. Results of my experiments reveal that adult worms, which are also expelled during evisceration, are unable to invade new hosts. Worms expelled during evisceration may be able to reinfect the holothuroid from which they came because the gut of the holothuroid would be open to the coelom. But unless reinfection of the old host occurs regularly, evisceration probably has a net negative effect on the worm populations.

The coelom of aspidochirote holothuroids is inhabited by gregarine protozoans and greatly modified gastropod molluscs belonging to the family Entoconchidae as well as by umagillid turbellarians (Barel and Kramers, 1977). Except for those species of entoconchids that maintain an open connection to the gut lumen or outside (reviewed by Lützen, 1979), evisceration has, until now, been the only known mechanism for release of infective stages of these parasites. The involvement of coelomic ducts in escape of infective stages of these groups of parasites from the coelom should be investigated in detail.

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INFECTION OF NEW HOSTS BY *ANOPLODIUM HYMANAE*, A TURBELLARIAN FLATWORM (NEORHABDOCOELA, UMAGILLIDAE) INHABITING THE COELOM OF THE SEA CUCUMBER *STICHOPUS CALIFORNICUS*

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ABSTRACT

Anoplodium hymanae, a member of the turbellarian family Umagillidae, parasitizes the holothuroid *Stichopus californicus* in the N. E. Pacific. Experiments revealed that the life cycle is direct, and that encapsulated embryos are the infective stage. Embryogenesis may or may not be completed by the time egg capsules pass out of the host of the parent worm. Developed embryos can survive in their capsules for 10–11 months, but they die if the capsules remain in sea water indefinitely. Hatching occurs when egg capsules that contain developed embryos are ingested by a sea cucumber; hatching is induced by the host digestive fluids. Larvae reach the coelom by penetrating the wall of the lower intestine or, more commonly, the wall of the respiratory trees. Larvae of *A. hymanae* and those of intestine-inhabiting umagillids differ in behavior, but appear similar in morphology. The size of worms infesting *S. californicus* varies seasonally and is correlated with the seasonal feeding behavior of the host.

INTRODUCTION

Most species of turbellarian flatworms are free-living, but more than 150 species belonging to more or less distantly related families have evolved symbiotic associations with other organisms (reviewed by Jennings, 1971; Shinn, 1984). The hosts are typically invertebrates. Symbiotic turbellarians may inhabit the outside surface, the digestive tract, the connective tissue, or the body cavity of the host. As far as is known, symbiotic turbellarians have simple life cycles that do not involve intermediate hosts. The life history has been elucidated for remarkably few symbiotic turbellarians, however (Jennings, 1971; Shinn, 1984). The life history is known for only one turbellarian that inhabits the body cavity of its host. That species, *Kronborgia amphipodicola*, belongs to the neorhabdocoel family Fecampiidae, and like most other fecampiids, inhabits crustaceans. The worms leave the host after attaining maturity. Females secrete a cocoon into which a large number of egg capsules are deposited. Free-swimming larvae hatch out, locate a prospective host, attach to the outside of it, secrete a cyst around themselves, and then bore through the body wall (Christensen and Kanneworff, 1965).

From information presented in taxonomic papers, it is evident that the reproductive biology of other groups of coelom-inhabiting turbellarians differs from that of the fecampiids. Members of other taxa typically produce and release egg capsules during the parasitic phase (Jennings, 1971, and references therein).

The largest of the families of symbiotic turbellarians is the neorhabdocoel family Umagillidae. These flatworms inhabit echinoderms and sipunculans (see Cannon,

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1982, for systematic review). Most of the 50+ described species of umagillids live in the intestine of the host, but ten species are reported to live in the intestine and coelom of sea urchins or sea cucumbers, and eight species occur exclusively in the coelom of sea cucumbers. All of the latter eight species belong to the genus *Anoplodium.*

Schneider (1858) reported that egg capsules of *Anoplodium parasita* are laid into the coelom of its hosts, *Holothuria* spp. He predicted that the egg capsules escape the coelom before hatching because egg capsules that he recovered from the coelom were never hatched. Schneider's hypothesis is correct for the related species *A. hymanae* (Shinn, 1985). Egg capsules of *A. hymanae* pass out of the body cavity of its host, *Stichopus californicus,* on a daily basis, apparently through ducts that connect the perivisceral coelom to the posterior end of the rectum. Egg capsules and adult or subadult worms are also cast out of the coelom when the hosts eviscerate. Thus, egg capsules and free-moving worms are potential infective stages for *A. hymanae.* Schneider (1858) hypothesized that embryos in egg capsules of *A. parasita* would hatch in the sea. Apparently he thought that free-swimming larvae penetrate new hosts, although he did not propose a site of penetration. Changeux (1961) presented the alternative hypothesis that encapsulated embryos of *A. parasita* are ingested by a new host, and that the embryos hatch in the intestine, then penetrate the wall of the intestine to reach the coelom.

This paper describes the mechanism of infection of new hosts by the umagillid *Anoplodium hymanae* Shinn. This flatworm and its host (*Stichopus californicus*) occur along the western coast of North America (Shinn, 1983a). The hypotheses of Schneider (1858) and Changeux (1961) concerning the mechanism of infection were tested experimentally, as was the hypothesis that free-moving worms that are expelled at evisceration can infect new hosts. The morphology of larvae is described. In addition, this study has revealed a distinct variation in the size of worms present in *S. californicus.* The variation is correlated with the seasonal feeding behavior of the host.

MATERIALS AND METHODS

Collecting sites, procedures for dissecting sea cucumbers, and the protocol for keeping worms alive outside of the host are described by Shinn (1985).

Maintenance of egg capsules to determine if embryos hatch spontaneously

Egg capsules that had been expelled by natural means from *Stichopus californicus* (see Shinn, 1985) were kept in loosely capped jars of filtered sea water. Most of the capsules were teased out of the masses of coelomocytes in which they had been expelled. The sea water was changed every 2–3 days. Approximately 400 large capsules (*i.e.,* the size produced by large worms) were kept in constant darkness, and about 400 large capsules were kept in an uncontrolled light/dark regimen. A separate batch of about 200 small capsules (the size produced by small worms) were also kept in an uncontrolled light/dark regimen. Subsamples of 20–50 capsules were examined with a compound microscope every week for the first two months, and then only every several weeks. Capsules in some of the subsamples were used in experiments concerning the stimulus for hatching (see below). During microscopic studies, coverglasses were supported with pieces of clay so that the embryos were not damaged.

Infection of new hosts by ingestion of egg capsules

Small *Stichopus californicus* (less than 5 cm long) were collected intertidally from Shaw Island, Washington (48°36′10″N; 122°48′80″W), where they had apparently settled as larvae and were growing up out of contact with adult *S. californicus.* Several specimens were dissected and found to be uninfested by *A. hymanae.* Consequently, two other specimens were used in experiments to test the hypothesis that new hosts are infected when they ingest capsules containing larvae. The experimental sea cucumbers were kept for several weeks in dishes of filtered sea water. They were fed a cereal-type baby food to sustain them and flush the digestive tract of inorganic debris. Periodic examination of the water in which they were kept revealed no egg capsules. Several dozen fully embryonated egg capsules of *A. hymanae* were added to the dishes over a period of 24 h. As feces were deposited, they were collected and examined for egg capsules. The sea cucumbers were dissected at the end of 48 h. They were rinsed thoroughly with fresh water, sliced open with a razor blade, and the coelom was flushed with filtered sea water. Washings from the coelom were examined for *A. hymanae.* The digestive tract/respiratory tree complex was excised and fixed in hot (60°C) Hollande's fixative, embedded in paraffin, and sectioned at 8 μm. Sections were stained with Weigert's hematoxylin and erythrosin B.

Site and cause of hatching

Fifty to seventy-five fully embryonated capsules of *Anoplodium hymanae* were pipetted into short pieces of the intestine of large *Stichopus californicus.* The pieces were tied at both ends in order to contain the digestive fluids. Two pieces were taken from the upper third of the intestine, which is characterized by a highly folded wall, and two were taken from the lower third of the intestine, which has a non-folded wall. The pieces of intestine were kept in autoclaved sea water and opened after 7 and 30 hours. After examination, the tissues were fixed, embedded in paraffin, and sectioned as described above. Similar results were obtained for the two durations, so the data were combined. The pieces of intestine were intact after seven hours, but the digestive epithelium had deteriorated by 30 hours.

Two sets of experiments were performed to determine whether hatching results from the direct action of the digestive fluids of *Stichopus californicus* on the opercular sutures of the egg capsules of *Anoplodium hymanae:* (1) intact capsules that had been treated to kill the enclosed embryos were submerged in digestive fluids from the upper, folded part of the intestine of *S. californicus.* The capsules had been collected after their natural release from the coelom and were kept in sea water until the embryos had completed development. The embryos were then killed by placing the capsules in a small amount of sea water and letting the water evaporate to about half its original volume. This required about 24 h. Some capsules became indented during the process, but they eventually returned to their original form. Before the experiments, the capsules were returned to normal sea water for several hours. They were examined with a compound microscope to verify that the embryos were dead and that the opercular sutures were intact. (2) Fully embryonated capsules were sliced open with a razor blade and the ends of the capsules that contained the intact opercular suture were immersed in digestive fluids of *S. californicus* as described above. To control for the effectiveness of the digestive fluids as "causing" hatching, capsules containing live, developed embryos were submerged in aliquots of the digestive fluids. The experiments were conducted at 10°C. The capsules were examined after 1 h and again after 18 h.

Infection of hosts by adult worms

Experiments to determine whether adult worms can infect new host are described in the Results section. The experimental sea cucumbers were kept in separate aquaria, without running sea water, so that worms introduced to the sea cucumbers could be recovered and counted. Although the objective was to determine if worms released at evisceration might be able to infect new hosts, worms used in the experiments were initially dissected from sea cucumbers. Unless sea cucumbers undergo some general physiological changes just prior to evisceration, the effect of being dissected from the host should not be much different for the worms than if they had been naturally eviscerated. This procedure was considered to be more natural than evisceration induced by injecting chemicals into the perivisceral coelom.

RESULTS

Fate of expelled egg capsules

Naturally released capsules of *Anoplodium hymanae* that were kept in sea water at local marine temperatures (8–12°C) contained developed embryos within three weeks of collection. The newly developed embryos were surrounded by a layer of yolk cells that had not been incorporated during embryogenesis.

Embryos did not hatch from either large or small capsules that were kept in sea water. This was the case for capsules kept in the dark and for those kept in an alternating light/dark regimen. Embryos in small capsules began to die after 5 months, but essentially all embryos in the subsamples of large capsules were alive after 8½ months. Approximately 80% of the large capsules contained live embryos after 10 months. After 11 months, the remaining 146 large capsules were examined: 58% (84) contained live embryos, and 42% (62) contained dead embryos. Some debris was visible in the capsules containing live embryos, but there were no recognizable yolk cells. I could not distinguish whether the yolk cells had disintegrated or had been consumed by the embryos. The walls of capsules that contained live embryos, or dead but intact embryos, were still pliable at the end of the eleven-month incubation period. The opercula could not be dislodged without destroying the capsules. Capsules that were compressed under a coverglass split lengthwise, though there was a tendency for the split to follow the suture for a short distance. Dead embryos eventually disintegrated in the capsules. The operculum of most capsules containing long-disintegrated embryos remained in place, but was usually dislodged by the slightest contact. No hatchlings were seen among any of the sets of capsules.

Infection of new hosts by encapsulated embryos

Fully embryonated capsules that were offered along with food to small, "worm-free" specimens of *Stichopus californicus* began to appear in the feces of the holothuroids after about 24 h. The feces had the form of elongate strings of detritus ensheathed by mucus. Egg capsules that had passed through the sea cucumbers were enclosed in the layer of mucus and were thus easily distinguished from capsules that had not been eaten. By the end of 48 h, 168 capsules had been recovered from the feces: 100 (60%) had the operculum dislodged and were empty; 7 (4%) were opened but still contained the embryos; 57 (34%) were closed and contained live embryos; 4 (2%) were closed but contained dead embryos. About 100 viable capsules were not ingested; none hatched.

Four small (150–220 μm long) *Anoplodium hymanae* were recovered from the perivisceral coelom of each of the two small *Stichopus californicus* to which the embryonated capsules had been fed. The worms were very hard to see because they were transparent and clung to pieces of tissue; additional small specimens may have been overlooked. In paraffin sections of the viscera of the sea cucumbers, numerous hatchlings of *A. hymanae* were observed among the food in the intestinal lumen. Several dozen worms in various stages of penetration through the wall of the lower part of the intestine and, especially, through the wall of the respiratory trees, were found in paraffin sections of one of the sea cucumbers (Figs. 1–4). Passage through these organs appears to occur in stages. Many specimens were located immediately beneath the luminal epithelium. This layer probably closes over the penetrating larvae very quickly because it was seldom observed to be broken. Specimens within the connective tissue layer of the respiratory trees and lower intestine were usually oriented parallel to the luminal and coelomic epithelia, with the ventral surface adjacent to the coelomic epithelium. No specimens extended completely across the wall of the respiratory trees or intestine.

Site and cause of hatching

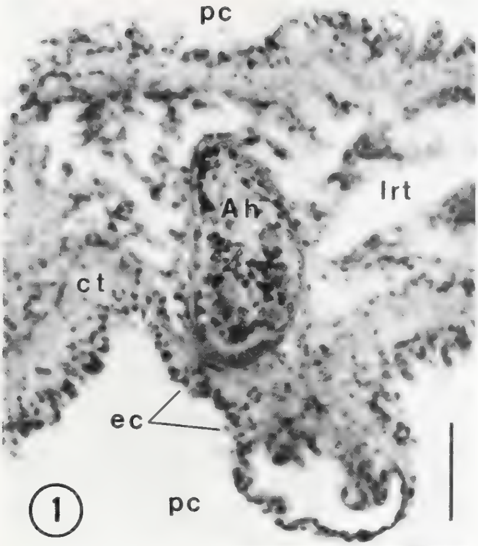
Fluid from only the anterior part of the intestine of *Stichopus californicus* is very effective at "causing" hatching of *Anoplodium hymanae.* Of 105 capsules that were introduced into tied-off pieces of the foregut of *S. californicus,* 82 (78%) had hatched, 3 (2.8%) were in the process of hatching, 17 (16%) were unhatched, and 3 (2.8%) were dead when the pieces were opened. Large numbers of larvae were recovered from the lumen of the pieces of foregut, but none was found to be penetrating the tissues when the latter were sectioned. Only two of 155 capsules introduced into pieces of hindgut hatched, though the embryos in the capsules were still alive at the end of the experiment.

Embryos hatched from 42 (86%) of 49 capsules that were immersed in fluids from the upper, folded part of the intestine of *Stichopus californicus.* These capsules constituted the controls for experiments to determine whether the opercular sutures of egg capsules are broken down by the enzymatic action of the intestinal fluids of *S. californicus* (see below). Three embryos had emerged by the end of one hour. At this time, 25 control capsules (not included in the above totals) were examined with a compound microscope: 6 had the operculum dislodged but still contained the embryo, and 19 had the operculum in place. To determine if the opercular suture of the intact capsules had become weakened in preparation for hatching, pressure was applied to the capsules by slowly drawing water out from beneath the coverglass. Slight compression resulted in the operculum being dislodged from 18 of the closed capsules; application of greater pressure caused the last capsule to split without the operculum becoming dislodged. The walls of the capsules were still pliant.

Fluids from the folded part of the intestine of *Stichopus californicus* did not weaken the opercular suture of capsules of *Anoplodium hymanae.* The experiments included 125 intact capsules whose developed embryos had been killed, and 20 capsules that were sliced open to allow the intestinal fluids to contact the inner edge of the opercular suture. The location of the opercular suture on the outer surface of the capsules was, however, more conspicuous after immersion in intestinal fluids. When the capsules were compressed after 18 h in the intestinal fluids the still-pliable capsules became deformed and eventually split without the operculum being dislodged.

Morphology of larvae

Larvae are dorsoventrally flattened and ovoid in outline when crawling over a firm surface. The body is colorless and completely ciliated. The larvae vary in size with the size of the capsule in which they develop, and with the number of zygotes that are incorporated into the capsule. The largest larvae are approximately 200 μm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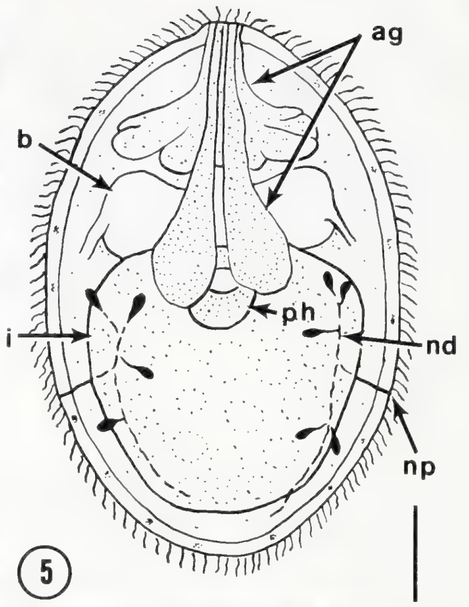
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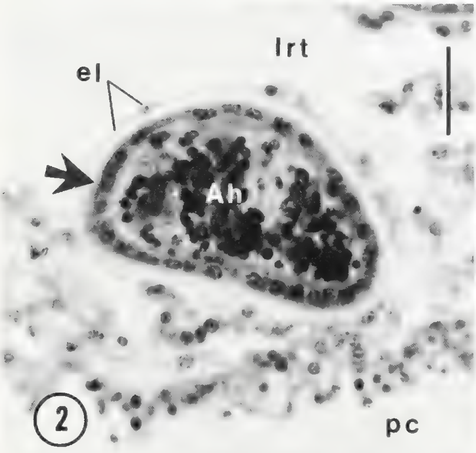
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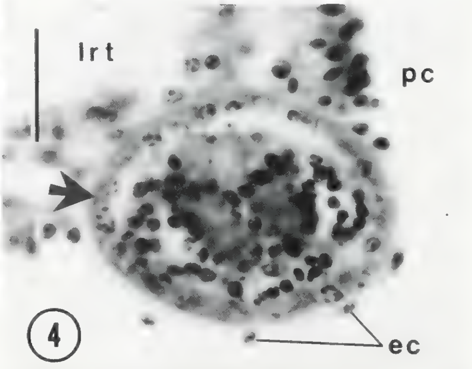

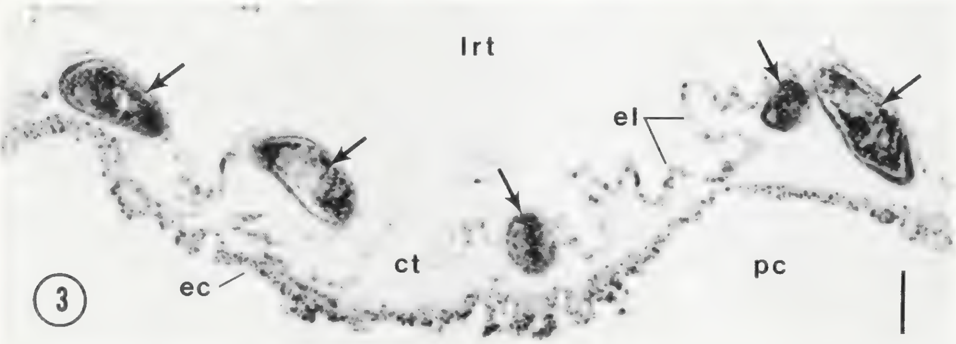
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FIGURES 1–4. Larvae of *Anoplodium hymanae* (Ah) in various stages of penetration through the wall of the respiratory tree of *Stichopus californicus* after experimental infection; light micrographs of paraffin sections; H & E stain. lrt, lumen of respiratory tree; el, luminal epithelium; ct, connective tissue wall of respiratory tree; ec, coelomic epithelium; pc, perivisceral coelom.

FIGURE 1. The worm is in contact at its anterior end with the luminal side of the wall of the respiratory tree. Scale = 50 μm.

long and 95 μm wide, but specimens as small as 70 μm long and 40 μm wide were seen. The epidermis consists of cuboidal cells that are approximately 5 μm tall; the cilia are about 5 μm long. The epidermal cells lack rhabdites. The epidermis remains intact during penetration of larvae into the coelom of the new host.

Newly hatched larvae contain at least four large, intensely eosinophilic gland cells which open at the anterior tip (Fig. 5; see also Shinn, 1985: Fig. 9). Two cells are positioned dorsally and extend ⅔ the length of the body. Two (possibly more) cells lie ventrally in the anterior ⅓ of the body. At least some gland cells were present in specimens that were penetrating the wall of the respiratory trees. The glands were lacking from even the smallest coelomic worms.

The cerebral ganglion lies immediately behind the ventral pair of anterior gland cells. It consists of a central bilobed neuropile surrounded by numerous cell bodies.

The mouth opens on the ventral midline about midbody. The doliiform pharynx measures about 20 μm in diameter, and is located immediately behind the brain. The pharynx leads dorsally to the intestine. The latter extends posteriorly and laterally to the edges of the body. It consists of large cuboidal cells which contain numerous, fluid-filled vacuoles, and lipoid and yolk inclusions.

The protonephridial system is arranged bilaterally near the lateral body margins. A nephridiopore is located on each side of the body just posterior to the level of the pharynx (Fig. 5). These lead into short, medially directed collecting ducts that divide into an anterior and a posterior duct. Five flame cells were visible on each side of the body; the tip of the posterior duct on each side apparently lacks a flame cell.

Can adult worms infect new hosts?

Adult *Anoplodium hymanae* that were exposed by various means to specimens of *Stichopus californicus* were never found to infect the sea cucumbers. Twenty worms were placed in aquaria that contained sea cucumbers. The worms were very active for at least several hours, but were all dead at the end of 24 h. They had been mutilated, apparently by the feeding activities of the sea cucumbers. Twenty worms were pipetted onto the dorsal epidermis of *S. californicus;* they quickly crawled off the holothuroids and eventually died on the bottom of the aquaria. Twenty *A. hymanae* were pipetted into the mouths of two *S. californicus;* the worms were necrotic when dissected out of the intestine after two hours. When specimens of *A. hymanae* were placed directly into digestive fluids that had been freshly removed from the anterior part of the intestine of a sea cucumber, they underwent several spasmodic contractions and died within seconds. Twenty worms introduced into the rectum of *S. californicus* via the inhalent current were expelled within an hour. The worms died on the bottom of the aquaria.

Intensity and seasonality of infestation

A census of *Anoplodium hymanae* infestations of 13–15 *Stichopus californicus* was made at five widely separated times during two consecutive annual feeding cycles

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FIGURE 2. Worm is located just beneath the luminal epithelium. The epidermis (arrow) of the larva is intact. Scale = 25 μm.

FIGURE 3. Sections through five worms (arrows), all located in the connective tissue compartment. Scale = 50 μm.

FIGURE 4. Worm (arrow) is located subjacent to the coelomic epithelium just before breaking out into the coelom. Scale = 25 μm.

FIGURE 5. Hatchling of *A. hymanae;* free-hand drawing from photographs and live specimens; dorsal view. ag, anterior glands; b, brain; i, intestine; np, nephridiopores; ph, pharynx; nd, protonephridial ducts. Scale = 25 μm.

of the host (Table I). During the first sampling period only the number and relative sizes of worms and the status of the host digestive tract was recorded in a systematic way. Subsequently, the lengths and reproductive status of the worms, and the number of egg capsules in the brown bodies were also recorded.

All *Stichopus californicus* examined during the first, third, and fifth sampling periods (*i.e.*, during the springs of 1981 and 1983 and summer of 1982) had fully differentiated digestive tracts filled with food. *Stichopus californicus* dissected during the second sampling period (during the fall of 1981) had either a degenerating intestine (lumen indistinct or filled with cellular debris) or a non-functional, regenerating intestine (lumen narrow and empty, regions of intestine not differentiated). Sea cucumbers dissected during the fourth sampling period (early winter of 1982) had a non-functional regenerating intestine or a regenerating intestine that contained a thin column of food. According to these observations, *S. californicus* does not feed between about early October and early December. Specimens in the population do not all cease and recommence feeding on the same dates, however. Only one specimen, which was collected during the fall of 1981 but which was not part of the *A. hymanae* census, had obviously eviscerated its digestive tract in the field.

Seventy-two of the 75 *Stichopus californicus* that were examined during the five sampling periods contained *Anoplodium hymanae* (Table I). The infested *S. californicus* contained a mean of 25.5 (range 1–147) worms. At all times of the year there was great variation in the intensity of infestation between hosts (Table I). The median number of worms per host was greater during the summer than fall of 1981, but the numbers of worms were not significantly different at the $P = 0.05$ level (Wilcoxon rank sum test). Conversely, the median number of worms per host was smaller during the summer than fall of 1982, though the numbers of worms were not significantly

TABLE I

Intensity of infestation of Stichopus californicus *with* Anoplodium hymanae *at different sampling periods*

Sampling period & date	Condition of intestines of *Stichopus californicus*	Percent of *Stichopus californicus* infested (no. examined)	Mean no. *Anoplodium hymanae* per host	Range
1. 9–24 June 1981	Functional, fully differentiated	93.7% (16)	17.5	0–52
2. 12 Oct. to 27 Nov. 1981	Non-functional	87% (16)	9	0–34
3. 23 Aug. 1982	Functional, fully differentiated	100% (15)	17.8	1–43
4. 4 Dec. 1982	Non-functional	100% (8)	26.4	1–106
	Functional, regenerating	100% (7)	52.7	1–133
5. 14 Apr. 1983	Functional, fully differentiated	100% (13)	44	4–147
Totals		96% (75)	25.5	0–147

different at the $P = 0.1$ level (Wilcoxon rank sum test). Over the entire study, hosts with a fully differentiated gut contained more worms than did hosts with a non-functional gut ($P < 0.05$, Wilcoxon rank sum test).

It was noted in an earlier paper that *Stichopus californicus* often contains two conspicuously different sizes of *Anoplodium hymanae* (Shinn, 1983a). This was the case for most hosts dissected during the spring and early summer (Table II). Each size class actually consisted of worms of a range of sizes; worms of intermediate sizes were generally absent (Fig. 6). Other *S. californicus* dissected during the five sampling periods contained worms within only a certain range of small sizes (Table II; Fig. 7). The percentage of hosts that contain only small worms apparently increases as the feeding periods progress so that during the late fall and early winter, *S. californicus* contain only small *A. hymanae* (Table II).

Seven *Stichopus californicus* that were dissected during August 1982 contained only small worms. Six of the seven contained capsules of the size produced by large worms. The embryos in many of the large capsules had not completed embryogenesis. Because the duration of the embryogenic period is 30–35 days (Shinn, 1985), the hosts must have contained large worms less than 35 days prior to dissection. Similarly, brown bodies in 7 of the 16 hosts dissected during October and November 1981, and 9 of the 15 hosts dissected during December 1982, contained capsules of the size produced by large worms, while at the time of dissection the hosts harbored only small worms. Most of the capsules contained viable, developed embryos. These observations provide further evidence that most hosts are inhabited by large and small worms early in the year, and that the large worms disappear from all hosts by the end of fall.

Over both years of this study, the mean number of egg capsules recovered from the coelom per host was greater during the spring and summer than in the late fall and winter (Table III). While large worms were absent from sea cucumbers during the winter, at least some small worms in some hosts were reproductive during the winter. Egg capsules are produced and, presumably, released to the environment for infection of new hosts at all times of the year.

TABLE II

Occurrence of large and small specimens of Anoplodium hymanae *in* Stichopus californicus *at different times of year**

Date**	Number of *S. californicus* examined	Percent of *S. californicus* infested with both large and small worms	Percent of *S. californicus* infested only with small worms	Percent of *S. californicus* that contained large capsules but no large worms
April (1983)	13	100%	0%	nd
June (1981)	16	63%	25%	nd
August (1982)	15	53%	47%	40%
Oct.–Nov. (1981)	16	0%	87%	43%
Dec. (1982)	15	0%	100%	60%

* See *Intensity and seasonality of infestation* section for detailed explanation of size classes of worms.
** Note that sampling dates from different years are arranged in a monthly sequence.

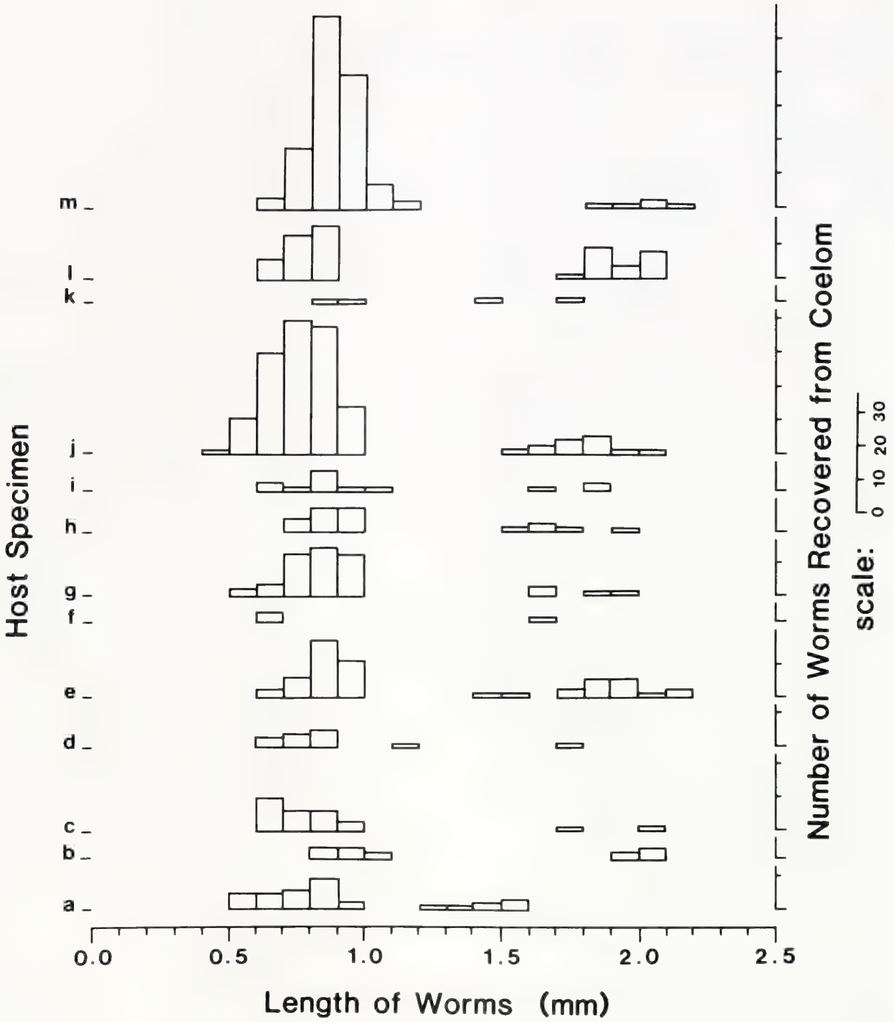

FIGURE 6. Histograms showing numbers of *Anoplodium hymanae* (ordinate) of various lengths (abscissa) recovered from each of 13 *Stichopus californicus* (a–m) on 14 April 1983. Most hosts contained worms of two size ranges, and lacked worms of an intermediate size range.

DISCUSSION

Infection of new hosts

It can be concluded from experiments and observations described in this paper that egg capsules containing developed embryos are the usual infective stage of the life history of *Anoplodium hymanae.* In contrast to Schneider's (1858) hypothesis, embryos do not normally hatch from capsules that remain in sea water. They hatch when the capsules are ingested by a potential host, as hypothesized by Changeux (1961).

Stichopus californicus is an epibenthic species. Like most other aspidochirote holothuroids, it consumes surface detritus by the use of its peltate, oral tube feet

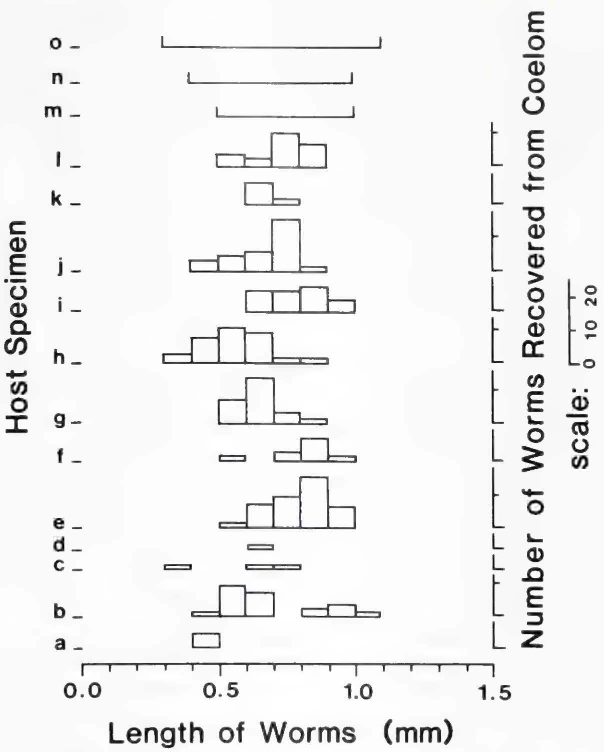

FIGURE 7. Histograms showing numbers of *Anoplodium hymanae* (ordinate) of various lengths (abscissa) recovered from each of 15 *Stichopus californicus* collected on 5 December 1982. Only the size range of worms are shown for hosts m, n, and o; they contained 122, 105, and 119 worms, respectively. The hosts contained only small worms.

(Massin, 1982; Cameron and Fankboner, 1984). Particles that stick to mucus on the feeding appendages are transferred to the mouth. Food particles might also become entrapped between small knobs of the oral tube feet (Cameron and Fankboner, 1984). The latter mechanism may provide some selectivity in feeding (Roberts, 1979). Egg capsules of *Anoplodium hymanae* are probably ingested at random along with detritus, although the long stalk on the capsules (see Shinn, 1985) may facilitate ensnarement of the capsules.

TABLE III

Seasonal abundance of egg capsules of Anoplodium hymanae *in brown bodies of* Stichopus californicus

Sampling period & dates	Total number hosts examined	Mean number capsules per host	Range in number capsules per host
1. 9–24 June 1981	1	763	—
2. 12 Oct. to 27 Nov. 1981	11	82	0–368
3. 23 Aug. 1982	15	487	2–3327
4. 4 Dec. 1982	14	287	0–1520
5. 14 Apr. 1983	13	969	161–2156

Twenty-seven of the 28 species of umagillids that have been described from holothuroids inhabit members of the order Aspidochirota (Cannon, 1982; Shinn, 1983a). The other species and an undescribed umagillid are reported from members of the holothuroid order Apoda (Barel and Kramers, 1970; Kawakatsu, 1983). Members of the Aspidochirota and Apoda are typically sediment-ingesting detritivores (Massin, 1982). No umagillids are known to inhabit dendrochirote holothuroids, which are typically suspension feeders. This restriction of both intestine- and coelom-inhabiting umagillids of holothuroids to detritivorous hosts suggests that the infective stages of these various species of worms are, like the egg capsules of *A. hymanae*, ingested along with detritus from the sea floor. The fact that *A. hymanae* reaches the body cavity via the digestive tract suggests that species of *Anoplodium* have evolved from intestine-inhabiting ancestors.

Developed embryos of *Anoplodium hymanae* hatch upon contacting digestive fluids in the foregut of *Stichopus californicus*. My experiments have revealed that the opercular suture of the egg capsules is not broken down by the digestive enzymes of the host. I conclude that weakening of the opercular suture results from the activities of the embryos themselves, and that the embryos are induced to hatch by the digestive fluids. Hatching of the intestine-inhabiting umagillid *Syndisyrinx franciscanus* is also apparently induced by the digestive fluids of its echinoid hosts (Shinn, 1983b). The identity of the hatching stimuli of these umagillids has not been investigated. *Anoplodium hymanae* and *S. franciscanus* are, in my experience, entirely host specific, yet hosts containing the two worms co-occur at the sites where animals for this study were collected. Investigation of the species specificity of the hatching stimuli could provide considerable insight into the mechanism by which host specificity is established. Unless the hatching stimuli are very specific, ingestion of egg capsules by non-host organisms, including animals other than echinoderms, would have adverse effects on the reproductive success of these umagillids.

The time between initial contact with fluids from the foregut of *Stichopus californicus* and emergence of the larvae varies considerably. Hatching probably occurs at various levels of the gut of new hosts. The larvae must burrow out of the column of food in the gut before they can penetrate into the coelom. Embryos hatching in small sea cucumbers reach the coelom mainly by passing through the wall of the respiratory trees. Most worms hatching in large hosts may bore through the wall of the lower intestine before reaching the level of the respiratory trees.

Glands opening at the anterior end of the oncosphere larva of the cestode *Hymenolepis diminuta*, and the oncomiracidium larva of the monogenean *Entobdella soleae* apparently secrete hatching enzymes (Holmes and Fairweather, 1982; Kearn, 1975, respectively). Similarly positioned glands in the larvae of the fecampiid turbellarian *Kronborgia amphipodicola* (Køie and Bresciani, 1973), the larva of the cestodarian *Austramphilina elongata* (Rohde and Georgi, 1983), and the miracidium of the digeneans *Fasciola hepatica* (Dawes, 1960; Wilson *et al.*, 1971) and *Fascioloides magna* (Coil, 1981) are thought to produce lytic secretions that aid in penetrating the tissues of their hosts. The anterior glands of *Anoplodium hymanae* are not obviously expired before or during hatching; if a hatching enzyme is secreted by this species, the anterior glands are probably not its source. The glands may secrete a lytic substance that aids in escape from the layer of mucus that ensheaths the food in the host intestine, or that aids in penetration into the coelom. Newly-hatched specimens of the intestine-inhabiting umagillid *Syndisyrinx franciscanus* also have anterior glands that disappear soon after hatching, but those worms do not bore through the tissues of their echinoid hosts (Shinn, 1983b). This suggests that additional functions exist for the anterior glands of umagillids.

Intensity and seasonality of infestation

The lack of worms of an intermediate range of sizes during the spring and summer reveals that there is a period during which *Stichopus californicus* is not being infected by *Anoplodium hymanae*. The cessation of feeding by *S. californicus* during the fall and early winter may cause the gap in sizes of worms. Small worms that are present before feeding stops presumably grow during the non-feeding period and would be considerably larger than the new worms of the next year. The magnitude of the gap in worm sizes will reflect the duration of the nonfeeding period plus the length of time between recommencement of feeding and ingestion of infective capsules. Additional gaps in sizes of worms will result from ingestion of egg capsules at widely separated times.

Because large specimens of *Anoplodium hymanae* are not present in *Stichopus californicus* during the late fall and early winter when hosts are not feeding, it can be concluded that the large worms grow up from small worms within a single year—presumably from the small worms of the previous feeding season. Death of large worms probably begins as early as June because all hosts dissected in April 1983 had large worms, and 5 of 16 hosts dissected in June 1981 lacked large worms. The recovery of much greater numbers of small worms (up to 133) than large worms (up to 22) from individual hosts suggests that there is also heavy mortality of small worms.

The lack of large worms during the winter cannot necessarily be explained merely by senescence of the worms that grew up during the previous fall nonfeeding period. Since egg capsules are released to the environment at all times of the year, *Stichopus californicus* can be infected at any time during the feeding period. Some small worms will be acquired from about December through early October. Because large worms are found as early as mid-April, small worms can apparently attain the large size in about seven months (Oct.–Apr.). Worms acquired early during the feeding period should be just attaining full size by the following nonfeeding period. Growth of the worms may be non-linear over time (*i.e.* there may be a limit on the growth of small worms acquired during a particular feeding period) or there may be a selective die off of all remaining large worms prior to the non-feeding season. The former hypothesis is perhaps more likely because the size range of small worms remains fairly constant through the spring and summer (*cf.* Figs. 6, 7).

Development and hatching of egg capsules in the coelom

Stichopus californicus may provide a variety of brooding services to *Anoplodium hymanae*. It is conceivable that encapsulated embryos take up nutrients from the coelomic fluid of the host. No studies of the permeability of umagillid egg capsules have been made, but the wall of egg capsules of some other parasitic platyhelminths is known to be permeable to small molecules including some amino acids and carbohydrates (Rowan, 1962; Wilson, 1967).

Some embryos of *Anoplodium hymanae* may hatch and mature in the coelom. While this would be difficult to demonstrate, its possibility is suggested by the observation of four embryos hatching from capsules that were freshly collected from the coelom by dissection, and by the recovery of some open capsules from the coelom. Alternatively, the embryos in the opened capsules may have died without hatching, the opercular sutures may have subsequently broken down and the capsules may have been cleaned out by host coelomocytes.

Embryos in many capsules complete development in the coelom and are infective as soon as they are released to the sea. This may reduce exposure of the capsules to various destructive phenomena during the relatively long developmental period during which the capsules are not infective. In contrast, the encapsulated embryos of *Syndisyrinx franciscanus* must complete their two-month embryogenic period in the sea before they are infective (Shinn, 1983b).

General conclusions

The life cycle of *Anoplodium hymanae* (Fig. 8) resembles that of the intestine inhabiting umagillid *Syndisyrinx franciscanus* in having fully embryonated capsules as the infective stage, and in having a simple life cycle (*i.e.*, no intermediate hosts are required; Shinn, 1983b). As far as is known, escape of infective stages of *A. hymanae* from the coelom depends upon the defense mechanisms of the host rather than specific adaptations of the parasite. The only major difference in the reproductive biology of

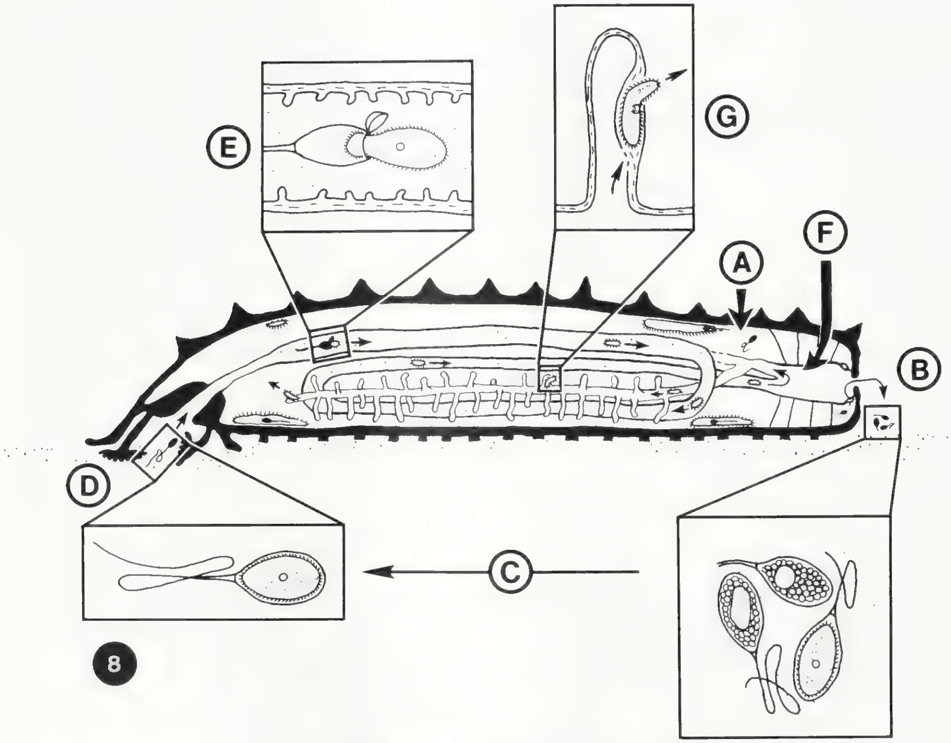

FIGURE 8. Life history of *Anoplodium hymanae*. A. *Anoplodium hymanae* releases egg capsules into the perivisceral coelom of the host. B. Capsules are encapsulated by host coelomocytes and collected into large masses called brown bodies. Brown bodies accumulate among suspensors of the rectum, then pass through ducts in the wall of the host's rectum and out the anus to the sea. At the time of release, encapsulated embryos are in various stages of development. C. Embryos complete development outside the host; developed embryos will survive in the capsules for many months but will not hatch if they remain in sea water. D. Egg capsules containing developed embryos are ingested as *Stichopus californicus* feeds on epibenthic detritus. E. Larvae hatch in response to digestive fluids in the upper intestine of the host; hatchlings escape the column of mucus-ensheathed detritus as it passes down the intestine. F. Larvae ascend the respiratory trees where the latter join the intestine. G. Larvae penetrate the wall of the respiratory trees and enter the perivisceral coelom of the new host.

these species that is associated with the difference in site of infestation is the ability of hatchlings of *A. hymanae* to penetrate the tissues of the host.

This study emphasizes that *Anoplodium hymanae* is fairly limited in the site of infestation in the host. Hatchlings of *A. hymanae* are not adversely affected by the digestive fluids of the host, but adult worms are killed by them. In addition, *A. hymanae* appears to have some means of avoiding attack by host coelomocytes. A fairly large number of species of umagillids are reported to inhabit both the coelom and intestine of the host (reviewed by Cannon, 1982; Shinn, 1984). Those cases should be carefully re-examined to determine if the worms clearly are adapted to inhabiting very different sites in their hosts, or whether the reports are the result of improper dissection techniques.

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REPRODUCTIVE BIOLOGY AND POPULATION STRUCTURE OF THE FIDDLER CRAB *UCA SUBCYLINDRICA* (STIMPSON)

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ABSTRACT

Comparatively large ova, low fecundity and low per capita egg production are adaptations to terrestrial habits in *Uca subcylindrica.* An unusual morphology of the genitalia appears to have co-evolved with the necessity for a lecithotrophic egg. Isolated populations are apparently sustained by rapid larval development. The crab stage population has a bimodal size-frequency distribution with an intermediate sex ratio pattern. Initially the sexes are equal, but males are more common in the larger size classes. Survival into the crab stage is relatively low. There is an increased rate of mortality with size. Only 30% of the individuals grow to modal carapace size; even fewer appear to participate in reproduction.

INTRODUCTION

Within *Uca,* the cosmopolitan genus of fiddler crabs, *Uca subcylindrica* (Stimpson) occupies a limited geographic range that coincides with the semi-arid zone near the mouth of the Rio Grande in the western Gulf of Mexico. Details of the species' biogeography and microhabit ecology have been described by Thurman (1984). This species is extremely terrestrial in comparison to other members of the genus. Other than taxonomic treatments (Barnwell and Thurman, 1984), few observations of the crab's natural history are available. Fisk (1941) and Peyton *et al.* (1964) reported the occurence of mosquito larvae in the burrows of *U. subcylindrica.* Preliminary observations of larval development were made by Thurman (1979) and extended by Rabalais and Cameron (1983). The present paper describes the reproductive biology and the consequence of anomalously rapid larval development on population characteristics of *U. subcylindrica.* Portions of this paper have appeared in abstract form (Thurman and Thurman, 1981).

MATERIALS AND METHODS

Uca subcylindrica were collected from 45 localities in south Texas and northeastern Mexico between 1972 and 1977 (Thurman, 1984). Six-hundred thirty-six (636) living specimens of this species were preserved in 70% ethanol. In addition, specimens of all other egg-laden fiddler crabs were collected. Egg diameter was estimated with the ocular micrometer of a stereo microscope by placing 1.0 mm-grid graph paper under a petri dish containing either fresh or preserved ova. Clutch sizes were calculated by counting the number of ova attached to a single pleopod of an egg-bearing female. This number was multiplied by the number of pleopods to obtain eggs per female (Gray, 1942). Larvae were reared from eggs in closed 4-liter plastic boxes containing 15‰ sea water as previously described (Thurman, 1979). Body length was measured in crab-stage individuals as the dorsal distance between the anterior and posterior

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margins of the carapace. Carapace width was measured as maximum distance between the anterolateral margins.

Standard deviations are given with means. Unless otherwise stated, significance was determined by Student's *t*-Test or Chi-square Test.

RESULTS

Egg size (1.0 ± 0.01 mm diameter) and the number of eggs in relation to female size (Table I, Fig. 1) compare closely to that reported by Rabalias and Cameron (1983). The authors reported a linear relationship between female carapace width (x) and the number of eggs (y) carried ($y = 81.77x - 914.99$; $r^2 = 0.80$).

Five ovigerous female *Uca subcylindrica* were captured accounting for only 1.5% of all mature females in the collection (Table I). *U. subcylindrica* were not collected in October, November, June, or July, however reproduction probably does not occur during these months. This is indicated by two observations. First, no ovigerous females were found in collections made between December and March. Second, in this area it rarely rains between June and early August and most surface waters become hypersaline and ultimately evaporate (Behrens, 1966; Thurman, 1984). Thus, eggs are probably produced only during the spring and late summer rainy periods. The low frequency of ovigerous females in the collection suggests they are rare in the population, even during breeding periods.

Other species of *Uca* were found to produce eggs between February and September. The microhabitat ecology of each species has been described elsewhere (Thurman, 1982, 1984). The egg diameters of all species except *U. subcylindrica* were similar (0.25 ± 0.02 mm). The earliest reproducing species was *Uca spinicarpa* Rathbun, which was collected with eggs in February. Ovigerous *Uca panacea* Novak and Salmon, *U. rapax* (Smith), and *U. longisignalis* Salmon and Atsaides were not captured until March. For all species except *U. subcylindrica*, ovigerous females were collected throughout the summer months, but the greatest frequency of egg-laden females per sampling occurred in late August and September. During summer peaks in reproduction, at least 20 to 30% of any population sample contained ovigerous females. Only 10% of the *U. spinicarpa* were collected in "berry." The apparently low frequency of berried-females in *U. subcylindrica* contrasts sharply with these brackish and fresh water species. A larger number of eggs is produced by those species living near brackish habitats.

TABLE I

Egg production in Uca subcylindrica *(Stimpson)*

Carapace width (mm)	No. ova	Collection date
16.3	50	30 April
18.4	500	15 April
20.6	960	15 August
22.9	1000	28 August
*23.3	—	25 May

* From the museum collection of the University of Texas Marine Science Institute at Port Aransas. This specimen, labeled as *Uca pugnax*, was collected from the Aransas Wildlife Refuge by J. W. Hedgpeth in 1946.

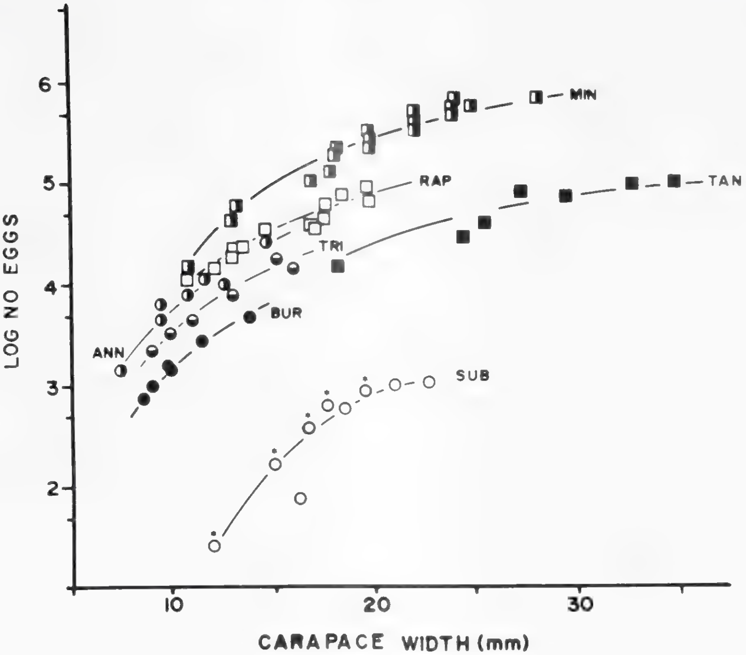

FIGURE 1. Variation in ova production by different species of *Uca*. Abbreviations: SUB = *U. subcylindrica*, n = 46 (○), BUR = *U. burgersi*, n = 6 (lb), TRI = *U. triangularis*, n = 5 (⊖), ANN = *U. annulipes*, n = 7 (◑), RAP = *U. rapax*, n = 25 (bb), TAN = *U. tangeri*, n = 7 (■), MIN = *U. minax*, n = 52 (◨). –*– indicates average for 1 mm width intervals from Rabalais and Cameron (1983).

In addition to season of egg deposition, the number of ova produced in each brood or clutch is of interest. Egg size is independent of female size. General brood characteristics have been reported for other Texas *Uca* (Rabalais and Cameron, 1983). The ova and clutch dimensions of *U. subcylindrica* are compared to allopatric congeners in Table II. The variation in number of eggs produced by different size classes in these species is shown in Figure 1. The data for *U. subcylindrica* are a combination of the counts in Table I and those provided by Rabalais and Cameron (1983). Ninety-five percent of egg production occurs by individuals with carapace lengths greater than 9.5 mm. In all species, as the size of the ovigerous female increases the number of ova carried per clutch becomes larger. However, there is considerable variation between species with respect to the maximum number of eggs carried by a female.

The distinctive structure of the male gonopodium and female gonopore found in *U. subcylindrica* is shown in Figure 2. Since the reproductive structure of other Gulf *Uca* have been published elsewhere, only a comparison with *Uca spinicarpa* is made here. Both male and female structures are much larger in *U. subcylindrica* than other species of *Uca*. The tip of the gonopod is about three times as broad while the aperture and genital operculum of the gonopore are seven to eight times larger in *U. subcylindrica* than those of other fiddler crab species. In the present examples, there are obvious physical restrictions associated with intraspecific reproductive compatibility.

The lecithotrophic egg in *U. subcylindrica* gives rise to rapidly developing larval stages. Larval development in most fiddler crab species lasts between two weeks and one month (Hyman, 1920; Herrnkind, 1968; Feest, 1969; Terada, 1979). Zoeal stages in *U. subcylindrica* last no longer than three days in culture (Thurman, 1979; Rabalais

TABLE II

Ova and clutch characteristic of Uca *from different habitats*

Species/reference	Habitat	$\bar{X}$ Female carapace width (mm)	$\bar{X}$ Ova radius (mm)	$\bar{X}$ Egg volume (mm^3)	$\bar{X}$ No. eggs brood	$\bar{X}$ Clutch volume (mm^3)
U. subcylindrica	Tr	13.4	0.5	0.52	627	327.3
U. burgersi (Gibbs, 1974)	ST	10.4	0.18	0.024	1782	42.8
U. triangularis (Feest, 1969)	IT	9.6	0.12	0.007	3990	27.9
U. annulipes (Feest, 1969)	IT-B	11.3	0.12	0.007	6400	44.8
U. rapax (Greenspan, 1980)	IT-B	15.8	0.13	0.009	28500	256.5
U. tangeri (Feest, 1969)	B-R	27.4	0.12	0.007	59000	413.0
U. minax (Gray, 1942)	R	20.3	0.13	0.009	184928	1666.3

Tr = terrestrial, ST = semiterrestrial, IT = intertidal, B = brackish, R = riverine.

and Cameron, 1983). There may be two or three instars before the megalopae appear. The megalopae metamorphose to the first crab stage within seven to ten days after hatching from the ova. The zoea do not feed. Megalopae, however, accepted brine shrimp. Some were observed to be cannibalistic.

Basic morphometric data were taken from crab-stages of the fiddler crabs. Carapace length and width in *U. subcylindrica* are closely correlated (Fig. 3). On a population basis, *Uca subcylindrica* appears to have the smallest average carapace length of all fiddler crab species in the region (Table III). Using carapace length, the size distribution of *U. subcylindrica* is illustrated in Figure 4. Over the sample, average carapace length is 8.1 ± 0.6 mm. However, the size-frequency distribution does not fit a single normal distribution ($P < 0.05$) by Chi-square test. Rather, these data are better described as a bimodal distribution. Subpopulation I possesses individuals wth carapace lengths less than 8.5 mm ($\bar{X} = 5.9 \pm 0.6$ mm). Larger individuals form subpopulaton II ($\bar{X} = 10.4 \pm 0.8$ mm). Each subpopulation fits a Poisson frequency distribution ($P > 0.05$; $Sub_I\ \chi^2 = 13.84$; $Sub_{II}\ \chi^2 = 12.08$). Within each there is no significant difference between mean carapace length in males and females. Individuals forming subpopulation I were segregated into discrete samples for each month between December and March. There are slight but insignificant differences in mean-monthly carapace length. Carapace length in subpopulation I increased by 1.0 mm between January and March. The carapace lengths of other species of *Uca* were unimodally distributed.

Across the entire population, two allometric abdomen forms are seen that are associated with puberty in female crustacea (Huxley, 1924; Hartnoll, 1974; Haley, 1969, 1973). Regardless of size, individuals having one enlarged cheliped, gonopodia, and a narrow abdomen were considered to be males. In the remainder of the population, individuals with two small chelipeds and sternal gonopores were considered females regardless of abdomen width. Some females have very broad while others possess narrow abdomens. In the lower portion of Figure 4, females with equal chelipeds but less than 5.5 mm carapace length all have narrow abdomens. The frequency of broadness increases as the carapace length reaches 8.0 mm. In larger size categories, all

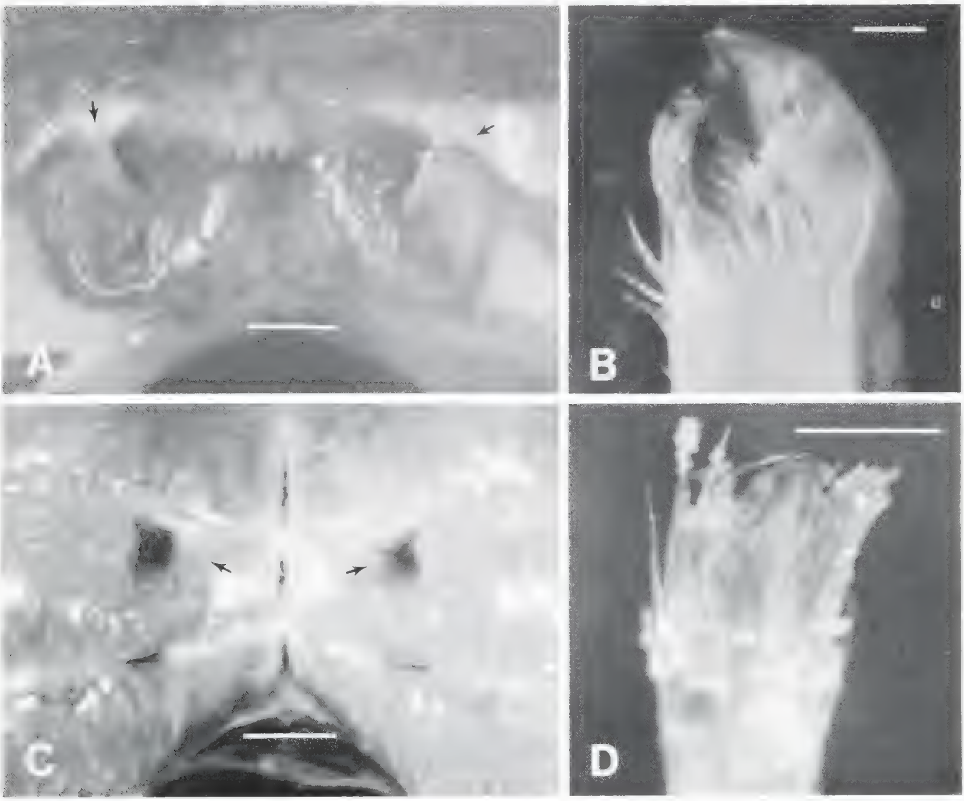

FIGURE 2. Morphology of the external reproductive structures found in Gulf *Celuca*. A. Gonopores of female *Uca subcylindrica*. Bar = 1.0 mm, arrows indicate genital tubercle. B. Posterior perspective of right gonopodium from male *U. subcylindrica*. Bar = 0.5 mm. C. Gonopores of female *Uca spinicarpa*. Bar = 1.0 mm, arrows indicates lateral margins. D. Lateral perspective of right gonopodium from male *U. spinicarpa*. Bar = 0.5 mm. Examples taken from individuals with carapace width between 17.0 and 18.0 mm.

females possess broad abdomens. The size intervals between 5.5 and 8.0 mm within subpopulation I represent a transitional phase to sexual maturity. This interval appears to represent morphologically but not functionally mature females since egg production is not observed until the crabs are in the 9–10 mm size category (Fig. 4).

Based on cheliped asymmetry and abdomen structure, the probability of maleness (P_m) averages 0.58 ± 0.10 (58%) over the entire population. In subpopulation I, the prepuberty sample, P_m = 53 ± 3%. This increases to 61 ± 4% in subpopulation II. P_m is calculated for each 1.0 mm length interval and compared throughout the population in Figure 5. Since the relative number of males increases with size, the sex ratio in the population is considered to be "intermediate" as described by Wenner (1972). A significant correlation coefficient of 0.727 is seen for the nine categories of carapace length ($P < 0.005$). The male:female ratio is between 1.8 and 2.3 in size classes greater than 8.0 mm. Since differential migration patterns between the sexes appears to be negligible in this habitat (Thurman, 1984), these data suggest that females either succumb to environmental pressures earlier or grow less rapidly than males after puberty as proposed by Fielding and Haley (1976) for other crabs. However, Colby and Fonseca (1984) have found reproductively active female *U. pugilator* to grow slower than males.

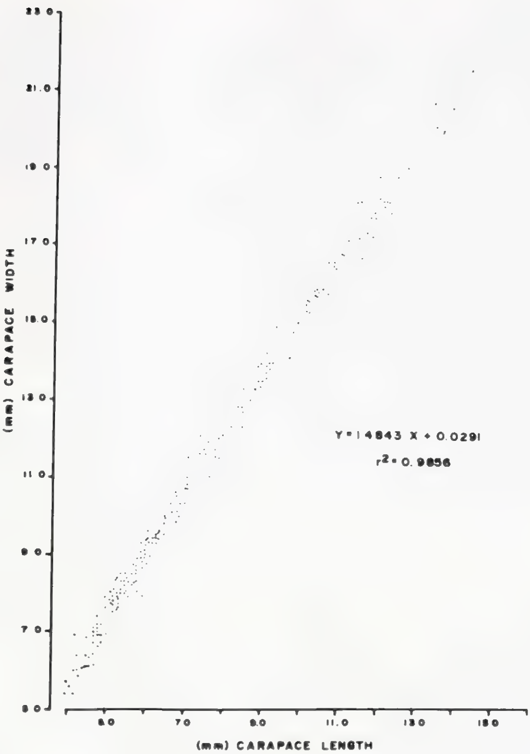

FIGURE 3. Relation between carapace length and width in *Uca subcylindrica* (n = 417). Linear regression calculated by least squares method for n = 150.

Survivorship curves can be used to compare the degree of adaptive success among species (Ricklefs, 1973). Assuming that size distribution represent losses from each morphological cohort due to death and/or growth, the logarithm of percent survival can be related to carapace size in four species of *Uca* that differ in microhabitat ecology

TABLE III

Relation between carapace length and width in Uca *from the western Gulf of Mexico*

Subgenous/ species	n	Carapace length ($\bar{X}$) (mm)	Carapace Width ($\bar{Y}$) (mm)	y = mx + b	r^2
Celuca					
subcylindrica	150	8.8 ± 2.9	13.4 ± 4.4	1.4843x + 0.0291	.9856
spinicarpa	156	10.5 ± 2.9	14.6 ± 3.1	1.4944x − 0.0263	.9768
panacea	125	10.0 ± 2.5	15.5 ± 3.5	1.4290x − 0.0191	.9879
Minuca					
rapax	123	10.5 ± 2.2	15.9 ± 3.4	1.5464x − 0.0315	.9789
longisignalis	74	11.5 ± 2.7	17.0 ± 4.0	1.4993x − 0.0283	.9851
vocator	19	9.7 ± 2.7	13.3 ± 4.1	1.5190x − 0.1416	.9952

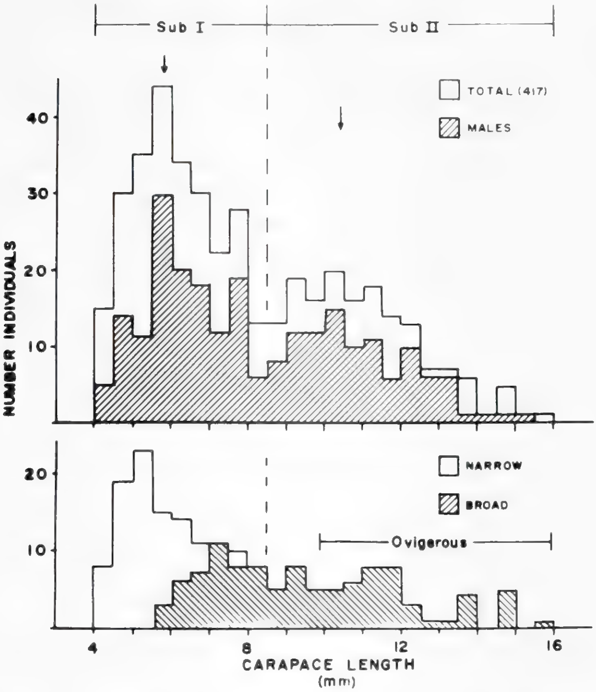

FIGURE 4. Size-frequency distribution of *Uca subcylindrica*. Upper graph: total/male component. Lower graph: female component. Sub I = subpopulation I, Sub II = subpopulation II. Arrows indicate mean carapace lengths for each subpopulation (5.9 ± 0.6, 10.4 ± 0.8 mm, respectively). Ovigerous size intervals indicated by bar in lower graph.

(Fig. 6). Since each grows to a different maximum size, absolute body length has been converted to a percent maximum size. The data for *U. burgersi* is taken from Gibbs (1974), *U. pugnax* from Huxley (1924), and *U. panacea* from Powers (1975). The patterns of survival can be identified among the basic schemes of Deevey (1947). They appear to be either type I where mortality is low except in larger size classes or type II in which mortality increases at a constant rate with size. A constant mortality rate is expressed as the linear-exponential line. Species that live in more mesic habitats, such as *U. pugnax* and *U. panacea*, have type I survivorship. More terrestrial species like *U. burgersi* and *U. subcylindrica* have type II curves. The *U. subcylindrica* curve is closer to being exponential than *U. burgersi*. Comparisons between species can be made by determining what portion of the populations achieve modal size categories. About 80% of *U. pugnax* and *U. panacea* survive to the modal-length interval. On the other hand, only 48% of the *U. burgersi* and 30% of the *U. subcylindrica* attain the same relative size. Mortality in the smaller size categories is greater in the terrestrial species.

DISCUSSION

Crustaceans have adapted to terrestrial habitats through various morphological, physiological, and behavioral mechanisms. Supralittoral and terrestrial life for brachyurans has been complicated by two major problems: (1) continuance of reproduction and development and (2) regulation of temperature, salts, and water (Bliss, 1968).

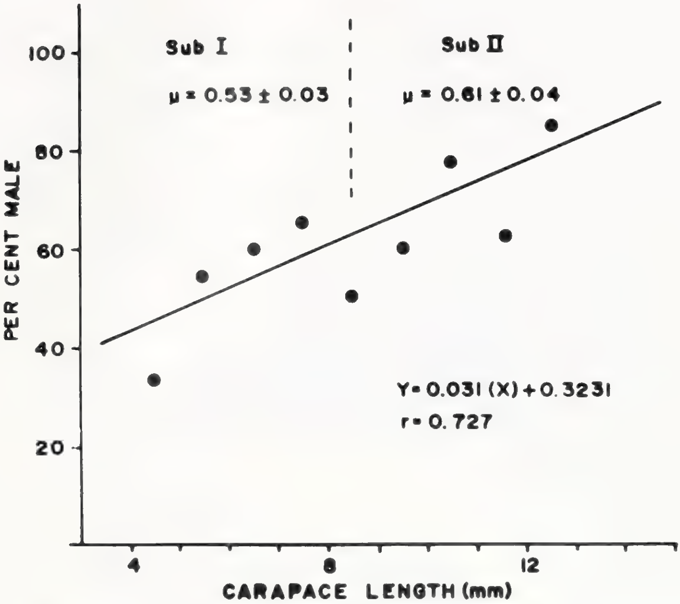

FIGURE 5. Percent males (P_m) *U. subcylindrica* in each 1.0 mm carapace length size interval. Linear regression computed by least squares method. Mean P_m for each subpopulation indicated.

Previously, Crane (1975) regarded the Red Sea as the most saline body of water inhabited by fiddler crabs. She reported the presences of three widely distributed Indo-Pacific species in habitats with salinities typically between 37 and 40‰. The *Celuca* appear to be the most successful of all subgenera in adapting to terrestrial habitats. From this terrestrial vantage point, *Uca subcylindrica* are unique among the fiddler crabs. Their limited geographic range is coincident with isolated lagoons in semi-arid south Texas and northeastern Mexico with salinities occasionally over 100‰ (Thurman, 1984). This species occupies very deep burrows near ephemeral bodies of water characterized by wide fluctuations in salinity up to 70‰ (*cf*, Hedgpeth, 1953). A continued survival of the species in this harsh habitat requires special modifications in reproduction. The reproductive biology of *Uca subcylindrica* contains anomalous features when compared to other species of fiddler crabs. Adaptations in egg production and larval development are ultimately reflected in the population structure of the species.

Early development

The terrestrial ecology of *U. burgersi* has been documented by von Hagen (1970b) and Gibbs (1974). This species is commonly found in habitats with salinities between 3.3 and 60.0‰, possesses extraordinary osmoregulatory abilities (Schmidt-Neilsen *et al.*, 1968), and has large eggs (0.36 mm dia.). The low ratio of ovigerous females observed in the present population sample (0.78%) is similar to the 6 females found by Gibbs (1974) in a sample of 535 *Uca burgersi* Holthuis. This statistic was estimated by a thorough transect and field study involving 45 crab populations in south Texas and Mexico (Thurman, 1984). The sampling of Rabalais and Cameron (1983) was systematically biased for ovigerous *U. subcylindrica* (n = 41) and could not be con-

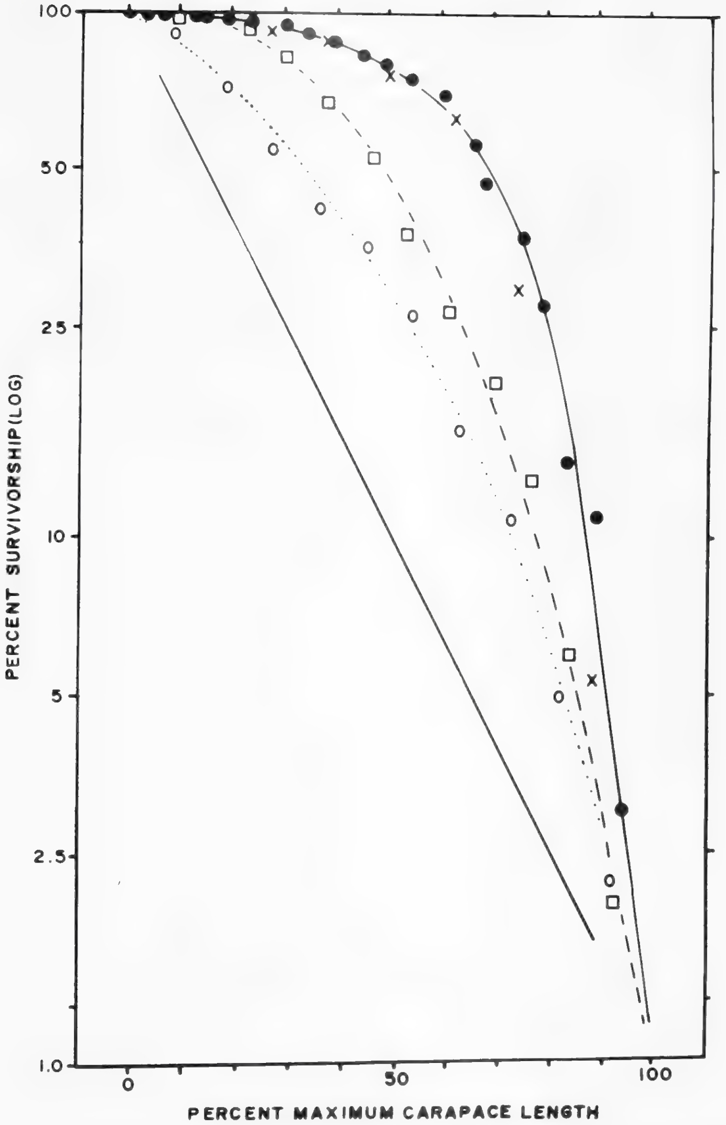

FIGURE 6. Survivorship curve adapted from Figure 4. *Uca subcylindrica* (○), *Uca pugnax* (●), *Uca panacea* (×), and *Uca burgersi* (□). All percents calculated for minimum to maximum size interval within each species. Linear line = constant exponential rate of decrease.

sidered in population estimates of reproductive potential. On Trinidad, the percent female *U. brugersi* with mature spermatheca is the lowest among nine species of fiddler crabs. Per capita frequency of reproduction is inversely correlated with terrestriality. Since *U. subcylindrica* is even more terrestrial than *U. burgersi* (Thurman, in press), it is not surprising that similar reproductive capabilities have evolved. The low fecundity and frequency of egg-production as well as large ova are parallel adaptations in the two ecologically similar species (Table II).

Early development in benthic marine invertebrates may vary along a spectrum from plankto- to lecithotrophy. Whatever the pattern, it represents the most energetically efficient strategy in producing the largest number of individuals surviving to produce eggs (Vance, 1973). When closely related species are compared, there is a fairly constant correspondence between egg size, developmental pattern, and size at metamorphosis (Christiansen and Fenchel, 1979). These characteristics appear to converge. The necessity for lecithotrophism will depend upon several abiotic factors. Generally, low food availability and temperatures are responsible for lecithotrophy in benthic polar and abyssl communities. However, as Thorson (1950) pointed out, at lower latitudes where predictable but short periods of rainfall occur, lecithotrophic development may occur in organisms with reproductive periods restricted to these seasons. Since reproductive periods in *U. subcylindrica* are synchronized with short rainy seasons, the latter strategy appears to be correlated with lecithotrophism in this subtropical species.

A broader perspective for fecundity in temperate and tropical *Uca* is outlined in Table II. Mean values for ovigerous female, ova, and clutch size are given for each species. The general habitat obtained from Crane (1975) for each is indicated along with an estimate of average ova and total clutch volume. Both size and number of eggs produced appear to correspond to environmental conditions. These are inversely related when intrageneric comparisons are made. Generally, riverine species possess the largest mean carapace width and carry the largest number of ova. On the other hand, the semi- and fully terrestrial species are smaller in body and clutch size. If one assumes the ova of each species are elementally similar, clutch volume may be an approximation of reproductive economics (Coe, 1949). For preliminary comparisons between species this is a reasonable measure since many details of the reproductive cycles for each species are not known. Although the eggs of *U. subcylindrica* are large (radius = 0.5 mm), clutch volume is well within the range of values calculated for other congeners. The apparent expense in producing an egg brood in the terrestrial species is no greater than that for female *U. rapax* or *U. tangeri* which carry a large number of smaller eggs. Consequently, there appears to be some certainty that a sufficient percentage of *U. subcylindrica* eggs will survive to reproduce as adults. To insure development, *U. subcylindrica* incubate their eggs for one to one-and-a-half months rather than 12 to 15 days as other *Uca* (Rabalais and Cameron, 1983).

As illustrated in Figure 2, the external genitalia of *U. subcylindrica* are large when compared to other *Uca*. Crane (1975) and Felder (1973) have used these as reliable taxanomic characters. However, these structures appear to possess little information on which to base any conclusions concerning phylogenetic affinity. Since survival in the semiarid habitats of this species has required the evolution of lecithotrophic embryos, the diameter of the gonopore has increases to allow oviposition of larger eggs. Since sperm transfer occurs by copulation in this genus, the large gonopod of the male has been selected to produce efficient intromission.

The abbreviated pattern of larval development in *Uca subcylindrica* provides two advantages in colonizing the semi-arid habitats with nontidal, ephemeral water found in south Texas (Thurman, 1984). First, larval success in settling depends upon the length of precompetent and competent periods, as well as predation rates. For invertebrates in general, the duration of each phase is correlated with the prevailing onshore and off-shore currents experienced during planktonic stages of development (Jackson and Strathman, 1981). In other *Uca*, larval release and settling are synchronized with local tides to facilitate dispersal and protection from planktovores (Christy, 1978, 1982; Wheeler 1978). The upper Laguna Madre has no well-defined tide or circulation pattern. Meteorological events are more effective in producing basin flushing than are predictable hydrographic forces (Smith, 1975). Since there is little prospect for oceanic dispersal of *U. subcylindrica* zoea, selection has minimized the duration of precompetent zoeal stages. Modifications such as this are known in the early life cycle of other crustacean species with limited dispersion potential. For example, the hymenosomatid crabs of western Australia assure recruitment into isolated populations by eliminating the megalopa stage of development. Rapid development in local nursery areas has evolved to compensate for low fecundity in these populations (Lucas, 1975).

This pattern is an advantage for a second reason. The length of time for zoeal phases is modified by diet and temperature. However, the duration of the megalopa stage is relatively constant in most *Uca* (Vernberg and Vernberg, 1975; Christiansen and Yang, 1976). In south Texas, brief periods of rain usually occur in May and September interrupting long periods of drought (Behrens, 1966). Since hypersalinity inhibits development (Rabalais and Cameron, 1982), egg deposition and rapid ontogeny in *U. subcylindrica* during wetter months is an optimal strategy capitalizing upon short but predictable fluctuations in an otherwise arid environment. Due to the compression of development time, the large, lecithotrophic larvae of this species do not require planktonic feeding. Consequently, non-planktonic development reduces predation mortality nearly to zero (Vance, 1973). Owing to the importance of osmotic stress over predation in this habitat, the anomalous development of this species compensates for both limited dispersal and physiological problems. If abiotic mortality factors are constant, rapid but isolated development ensures recruitment in an inhospitable habitat. In a discussion of tropical gastropods, Vermeij (1978) has suggested that marine species inhabiting lagoons and isolated inshore habitats tend to develop as endemic forms while more planktonic species are cosmopolitan. This observation appears to have some application in understanding the evolution of *U. subcylindrica* and the general level of fiddler crab endemism in the Gulf of Mexico (Barnwell and Thurman, 1984). This accelerated ontogeny has a pronounced effect on crab population size-structure.

Population structure

The structure and sex ratio in populations of *U. subcylindrica* can be compared to published data on more widely distributed crustaceans and fiddler crabs. The population structure of this species is more similar to crustaceans from higher latitudes than less terrestrial members of its own genus. The size-frequency distribution of a population is a dynamic characteristic that can change throughout the year as a result of reproduction and rapid recruitment from larvae. Several investigations have observed unimodal population size-structures in more slowly developing species of *Uca*. These include *U. pugilator* at Woods Hole (Huxley and Callow, 1933), *U. panacea* (Powers, 1975), and *U. pugnax* (Huxley, 1924) from the temperate zone where reproduction occurs with intensity between July and September. More southerly population of *U. pugilator* exhibit a progression in modal size-frequency distribution throughout the reproductive season (Colby and Fonseca, 1984). Tropical populations of *U. rapax*, *U. cumulanta* (Ahmed, 1976), and *U. burgersi* (Gibbs, 1974) are also unimodally distributed by size class. These species appear to reproduce more or less continuously throughout the year. On the other hand, the impact of recruitment from rapidly developing larvae has been reported in boreal crabs. The size frequency distribution of the crab stage in *Pisidia longicornis* is bimodal. Population recruitment occurs on an annual basis and development takes only three to four months (Samuelson 1970;

Smaldon 1972). The larger "adult" component is stable throughout the year. Smaller size categories correlate with larval settlement and growth processes throughout the year. In *U. subcylindrica,* body-size is bimodally distributed as a result of lecithotrophism and accelerated development. Growth of larvae into crabs in less than two weeks produces rapid seasonal recruitment into smaller size classes before ephemeral nursery grounds can evaporate.

Sex ratios have been reported for several species of fiddler crabs and appear to be related to the system of mating used by each (Christy and Salmon, 1984). Most exhibit the intermediate sex ratio pattern described by Wenner (1972). Powers (1975) found the percent males (P_m) to average 63% for *U. panacea* and 52% for *U. rapax* (= *U. virens*) in south Texas. *U. pugilator* in North Carolina has an average P_m of 39% (Colby and Fonseca, 1984). Along the temperate Atlantic coast, *U. pugnax* populations may vary in P_m from 53% in Georgia (Wolf *et al.*, 1975) to 59% in Massachusetts (Valiela *et al.*, 1974). This may be increased to 83% following an ecocatastrophe (Krebs and Burns, 1977). This species differs from others by having a P_m that decreases with size class. In tropical Venezuelan populations, the P_m of *U. rapax* is 59% while *U. cumulanta* is 54% (Ahmed, 1976). *Uca burgersi* populations on Barbuda have a P_m of 67% (Gibbs, 1984). The ratio of 58% males observed in *U. subcylindrica* is similar to the frequency observed in other *Uca* populations; the P_m increases with the size category just as in its tropical relatives.

Few females appear to survive into the larger size categories. Less than 25% of the entire population survives to size of first reproduction (9.5 mm body length). Although selection has reduced ontogeny and increased ova size, a delay in ova production to later in life can decrease the reproductive capacity of a species since mortality increases with size. However, all measurements of reproductive success are higher for individuals with a delayed onset of reproduction (Schall and Leverich, 1981). In fiddler crabs, larger individuals produce larger egg broods and apparently experience greater success in finding mates (Greenspan, 1980). In *U. subcylindrica* populations, rapid development and a delay in size of first reproduction may be balanced against the respective risks of mortality owning to abiotic factors. This overall reproductive strategy would assure a high return on reproductive investments and population recruitment in an inhospitable environment. This is accomplished without creating an excessive reproductive load with a large volume of unsuccessful offspring.

In conclusion, the single greatest stimulus to fiddler crab evolution is the presence of an unoccupied habitat (Crane, 1975). Specializations within the genus have led members to radiate in three directions: (a) into more terrestrial habitats, (b) to a greater partitioning of existing habitats between co-existing species, and (c) to increased sociality. Crane remarked that the unusually round body and striking reproductive appendages of *U. subcylindrica* are useful for its identification. In order to occupy more terrestrial habitats, the crab carapace has evolved as an arched, semi-cylindrical structure with increased volume. In addition, the more terrestrial representatives have broader frontal regions and short eyestalks and orbitals when compared to amphibious relatives (von Hagen, 1970a). Generally, less tomentose legs and carapace are found in more recently evolved terrestrial species (von Hagen, 1970b). The pubescence and long setae in *U. subcylindrica* appear to be adaptations for acquisition of substrate water in a dry environment as in other species (Thurman, 1982, 1984; Wolcott, 1984). Consequently, the tomentum in this species is a more highly evolved rather than a primitive characteristic. Maxillipeds, genital armature, acoustical signaling, combat behavior, and reproductive physiology are additional modifications associated with terrestrial adaptation (von Hagen, 1970c, 1975; Crane, 1975). Structure of the reproductive apparatus reflects a selective response to large ova and mating in a semi-arid habitat. The body structures and reproductive armature reflect ecological adaptation rather than phylogenetic affinity.

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PHOTOSYNTHESIS AND RESPIRATION IN *TRIDACNA GIGAS* AS A FUNCTION OF IRRADIANCE AND SIZE

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ABSTRACT

The effects of irradiance level and size on the rate of O_2 evolution and consumption was examined in *Tridacna gigas* using an oxygen electrode. Seven photosynthesis-irradiance (P-I) curves were generated for intact clams ranging from 1 to 23 cm in shell length. Both alpha and P_{max} decreased with increasing size of the clam. Oxygen evolution at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ and consumption in the dark were measured for an additional 9 clams ranging up to 38 cm in shell length. Oxygen evolved per gram clam tissue in the light, dark respiration, and the number of zooxanthellae per gram all decreased with increasing clam size. The average amount of shading experienced by the zooxanthellae in the clam tissues was estimated by comparing the P-I curves of different-sized clams to the P-I curves of zooxanthellae freshly isolated from these clams. Estimates of shading increased from negligible levels in small clams to 80% in the largest clams investigated. Use of these results to chose optimum light intensities for the mariculture of *T. gigas* is discussed.

INTRODUCTION

Bivalves of the family Tridacnidae are found throughout most of the Indo-West Pacific (Rosewater, 1965), although populations of the larger species have been declining in some areas in the face of intense overfishing (Pearson, 1977). One of the characteristics of tridacnid bivalves is their symbiosis with algae, and it has been suggested that the association may be a factor contributing to the large size of some tridacnids (Yonge, 1980), the largest of which, *Tridacna gigas,* has been observed to reach a length of 137 cm (Rosewater, 1965). The symbiotic alga *Symbiodinium microadriaticum,* occurs predominantly within the hemal sinuses of the hypertrophied siphon (commonly referred to as "mantle tissue" in the tridacnids) (Fankboner, 1971; Trench *et al.,* 1981). Only a few other marine bivalves are known to harbor symbiotic dinoflagellates, *e.g.,* the heart shell *Corculum cardissa* (Kawaguti, 1950).

The use of the larger tridacnids as food, and the resultant overfishing has generated an interest in the possibilities of commercial cultivation (see Munro and Heslinga, 1982). In the context of mariculture, studies of these clams have followed three directions. First, attempts have been made to gain an understanding of the spawning of clams with an aim towards controlled induction of spawning (Wada, 1954; LaBarbera, 1975; Jameson, 1976; Fitt and Trench, 1981); second, the artificial rearing of fertilized eggs through the developmental stages of metamorphosis and acquisition of algal symbionts (Beckvar, 1981; Fitt and Trench, 1981; Gwyther and Munro, 1981; Fitt *et al.,* 1984); third, an analysis of the importance of the contributions of the symbiotic algae to the nutrition of the clams (Muscatine, 1967; Goreau *et al.,* 1973; Trench *et al.,* 1981). This last parameter is important, since the potential for a significant proportion

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of the nutrition of the clams being provided by the symbionts renders these clams distinct from other bivalves that have been central to mariculture attempts in the past (Munro and Heslinga, 1982).

Previous studies (Wells *et al.*, 1973; Johannes *et al.*, 1972; Trench *et al.*, 1981; Mangum and Johansen, 1982) on the physiology of tridacnids and their zooxanthellae were not designed to yield data which could be directly applied to mariculture. Specifically lacking from the literature is information on the physiology of *T. gigas,* a prime candidate for mariculture (Munro and Heslinga, 1982) and information on how the physiology of the tridacnids change with age. *T. gigas* was chosen for this study not only because of its importance in the context of mariculture, but also because of some features which facilitated this work: the wide size range of individuals available makes possible a study of the relation between a variety of physiological parameters and the size of the clam; adults are not attached to the substrate (unlike *T. crocae* and *T. maxima*) and therefore collecting and placing them in a respirometry chamber can be accomplished with a minimum of stress; the majority of individuals of all sizes open soon after placement in a chamber and appear to behave normally throughout the experiment (unlike most other tridacnids).

P-I relations have been investigated in a number of associations between zooxanthellae and marine invertebrates (Barnes and Taylor, 1973; Scott and Jitts, 1977; Falkowski and Dubinsky, 1981; Fitt *et al.*, 1982). Similarly P-I relations of freshly isolated zooxanthellae have been described using zooxanthellae from a variety of invertebrates (Zvalinskii *et al.*, 1980; Dustan, 1982; Trench and Fisher, 1983). This study is one of only a few in which the P-I relations of the zooxanthellae *in situ* and *in vitro* are compared (Crossland and Barnes, 1977; Chalker and Taylor, 1978; Muller-Parker, 1984). Only Crossland and Barnes (1977) demonstrated shading of the zooxanthellae *in situ* (in the coral *Acropora acuminata*). In this study the effects of *in situ* shading on the zooxanthellae in a wide size range of clams are investigated. The large range in the thickness of the mantle (siphonal) tissue (where most of the zooxanthellae are situated) makes *Tridacna gigas* ideally suited for such a study.

MATERIALS AND METHODS

Aquisition and maintenance of Tridacna gigas

This study was conducted at the Micronesian Mariculture Demonstration Center (MMDC) in the Republic of Belau (Palau), West Caroline Islands, Micronesia, between May and July, 1983. The three largest clams used in this study (wet weight 425–1700 g, shell length 23–38 cm) were collected on the reef at depths of 1.5 to 3.5 m. After collection the animals were maintained at 4 m depth on the reef adjacent to the laboratory. All of the smaller clams used in this study were reared at MMDC by G. A. Heslinga and F. E. Perron (Munro and Heslinga, 1982). Surfaces of the shells of the clams were cleaned using a scalpel and toothbrush to remove epiphytic and epizooic growth from the shell and then maintained for between two days and two weeks in unfiltered flowing sea water in a concrete raceway exposed to ambient insolation. These raceways have been used to rear giant clams for up to three years at MMDC (Heslinga *et al.*, 1984).

Intact clam oxygen exchange

The respirometer chambers were constructed of glass and clear lucite and fitted with clear lucite lids. The volume of the four chambers used in this study ranged from 20 ml to 61 l. All four chambers contained a stir bar mounted directly below the oxygen electrode. During its operation, the stir bar maintained an adequate flow of water across the membrane of the electrode. This was sufficient to mix the water in the three smaller chambers, but in the largest chamber an additional larger stir bar was used. The three smaller chambers were water-jacketed in order to maintain a constant temperature of 28°C. The largest chamber was not jacketed, but no temperature variation was observed during any experiment.

A YSI model 53 oxygen electrode coupled to a Cole Palmer model 8377-15 chart recorder was used to record the change in oxygen tension within the chamber. Illumination was provided from above by a Mini-Cool movie light, and the irradiance was varied from 25 to 3000 $\mu E \cdot m^{-2} \cdot s^{-1}$ by adjusting the light-to-chamber distance. Irradiance was measured with a Li-Cor quantum photometer model LI-185 and a model LI-192S submersible quantum sensor.

Before placing a clam in a respirometer, the shell was lightly brushed to remove any growth accumulated since the original cleaning. It was then rinsed with 0.45 μm (pore size) filtered sea water and placed in an appropriate respirometer containing 0.45 μm filtered sea water. All rate measurements were made between 80–100% of air saturation using a linear portion of the trace after at least 10 minutes of equilibration time under each condition. Only data obtained from clams with fully expanded mantles are reported in this study. When investigating clams smaller than 2 cm in shell length (wet weight < 0.05 g), 2–6 individuals of the same size were placed in the chamber in order to achieve easily measurable rates of oxygen exchange. These experiments were always conducted between 10:00 a.m. and 5:00 p.m., and the order of the different irradiances was random. Dark respiration rate measurements were obtained between every irradiance level by turning out the light and covering the chamber with black plastic.

As a control for oxygen flux not due to *T. gigas*, measurements were made with both empty chambers and chambers containing clam shells from which the animals had been removed. No oxygen change was detected at any light level in the two larger chambers or at irradiances up to 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the smaller chambers. A small change was observed in the smaller chambers at the higher irradiances, both in the presence and absence of empty shells, and was subtracted in the rate calculations. This small change was presumably due to the effect of temperature on the oxygen electrode when the movie light was in close proximity to the small volume chambers.

Clam vital statistics

Shell dimensions (length, width, height) were measured to the nearest millimeter with calipers and total volume, by displacement. The clams were dissected from their shells and their total wet weight (blotted dry) measured to the nearest milligram. The clams larger than 2 cm in shell length were then further separated into three tissue classes (muscle, mantle, and remains) and weighed. The mantle and remains were homogenized separately in known volumes of filtered sea water (0.45 μm pore size millipore filter) in a Virtis tissue homogenizer. Replicate samples of each homogenate were removed and prepared for analysis of chlorophyll as described below. In the case of clams smaller than 2 cm, the entire clam was homogenized.

Isolation of zooxanthellae

Zooxanthellae from larger clams were isolated from a portion of the mantle homogenate described above, following the methods of Trench (1971a) (in the case of clams smaller than 2 cm, zooxanthellae were isolated from the whole animal homogenate). The homogenate was first filtered through three layers of cheese cloth to remove pieces of animal debris. Zooxanthellae were then isolated from the resultant slurry by repeated 2-minute centrifugations at approximately 1000 rpm in a Beckman table top centrifuge. The cells were resuspended in filtered sea water between each centrifugation. This procedure was repeated three times, after which the supernatant was clear and very little debris was observed upon microscopic examination of the pellet. The cells were resuspended in filtered sea water to approximately 10^6 cells/ml and the concentration determined by 6 replicate counts in a hemacytometer. Two aliquots (usually 5 mls) of this suspension were removed for analysis of chlorophyll and the remainder was used for analysis of photosynthetic and respiratory oxygen exchange by isolated zooxanthellae.

Quantification of chlorophyll

The samples were first pelleted at approximately 2500 rpm for 2 min and the supernatant discarded. The pellets were then resuspended in 0.4 ml of distilled water and frozen and thawed three times over a 24-hour period. The thawed samples were then ground in a Tenbroeck ground-glass tissue grinder with 3.6 ml of acetone and allowed to extract overnight in the dark at 0°C. The samples were clarified by centrifugation at approximately 2500 rpm and the absorbance of the supernatants were measured at 630 and 663 nm in a Turner model 330 spectrophotometer. The pellet was refrozen in 0.4 ml of distilled water, and re-extracted with 3.6 ml of acetone and the absorbance measured as before. The total amounts of chlorophylls *a* and c_2 in the samples were calculated using the equations of Jeffrey and Humphrey (1975). Replicate samples usually agreed within 10% and the average of the two replicates was used for further calculations.

Oxygen exchange by isolated zooxanthellae

Measurements of photosynthetic oxygen evolution were made in a YSI stir bath chamber using a YSI model 53 oxygen electrode coupled to a Cole Palmer model 4377-15 chart recorder. Temperature was maintained at 28 ± 0.5°C with a recirculating water bath. Illumination was provided by a Viewlex model V-25-P slide projector and irradiance was varied from 15 to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ by placing plastic window screen over the projector lens and varying the projector-to-chamber distance. Irradiance levels were measured using a LiCor Quantum photometer with a submersible quantum sensor. Dark respiration rates were determined when the incubation chamber was covered with black plastic. Freshly isolated zooxanthellae were suspended in filtered sea water at densities of 10^6 cells/ml in order to minimize self shading by the algal cells. Incubation volume was 3.0 ml as suggested by the instrument manufacturer. In experiments where photosynthetic oxygen evolution at more than one irradiance level was measured (for the photosynthesis *versus* irradiance curves), fresh cells were placed in the chamber after every other measurement to minimize any deleterious effects of the stir bar on the cells. In order to reduce variation due to possible photosynthetic rhythms (Prézelin *et al.*, 1977; Chalker and Taylor, 1978; Muller-Parker, 1984), experiments were conducted between 10:00 a.m. and 4:00 p.m. and the order of the different irradiances was random.

RESULTS

The 33 clams used in this study ranged in shell length from 0.86 to 38 cm, and in wet weight from 0.014 to 1700 grams (Table I). The allometric relation between

TABLE I

Daily P/R and CZAR in Tridacna gigas

Wet weight w/o shell (grams)	Shell length (cm ± S.D.)	Daily P/R 500 $\mu E \cdot m^{-2} \cdot s^{-1}$	Daily P/R 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$	%CZAR 40% trans.	%CZAR 95% trans.
0.014	1.0 ± 0.08 (4)	1.4	1.5	63	149
0.02	1.15 ± 0.05 (6)	2.4	2.5	109	259
0.041	1.55 ± 0.01 (4)	3.0	3.2	134	318
0.045	1.84	1.05	1.1	60	142
0.052	1.54 ± 0.05 (6)	2.7	2.8	120	284
0.08	2.12	1.4	1.5	64	152
0.84	4.0	1.55	1.7	72	169
1.0	4.3	1.65	1.8	76	179
1.25	4.8	1.55	1.7	68	164
1.325	6.5	1.4	1.6	67	160
14.79	10.0	1.3	1.7	70	167
18.2	10.3	2.3	3.1	128	312
290	23.5	1.5	2.0	105	249
424.6	23.0	0.82	1.1	44	112
1700	38	1.05	1.4	60	142
Average ± S.D.		1.67 ± 0.6	1.9 ± 0.65	83 ± 27	197 ± 65

P/R was calculated assuming 10 hours of sunlight at either 500 or 1000 $\mu E \cdot m^{-2} \cdot {}^{-1}$ and 24 hours of respiration. CZAR was calculated using the following equation and the translocation values indicated in the table.

$$\text{CZAR} = [\text{PG}(1000\ \mu E \cdot m^{-2} \cdot s^{-1}) - 0.05\text{R}](10\ \text{h})(\%\text{Translocation})(100) + \text{R}(24\ \text{h}).$$

the wet weight of the clam (without its shell) and the length of the shell is described by the following equation:

$$\text{Shell Length (cm)} = 4.246\ (\text{wet weight in g})^{0.30}$$

This equation was calculated in the log form:

$$\log(\text{shell length}) = 0.3 \log(\text{wet weight}) + 0.628 \qquad (r = 0.995,\ n = 16)$$

(Correlation coefficients for all allometric equations refer to the log transformations.)

The amount of chlorophyll *a* per gram of clam, as well as the number of zooxanthellae per gram of clam decreased with increasing size of the clam (Fig. 1). Chlorophyll *a* ranged from 2.0 to 3.1 pg/zooxanthella cell in the various clams but did not vary as a function of clam size. Weight of the "mantle" tissue ranged from 27 to 55% of the wet weight of the larger clams (clams smaller than 0.1 g wet weight were not dissected into the three tissue classes). An average of 87% (±6.2%, n = 8) of the chlorophyll *a* was found in the "mantle" tissue of the larger clams.

Initial observations indicated that while the smallest size-class of clams (<2.0 cm) demonstrated saturation of oxygen production at 600 $\mu E \cdot m^{-2} \cdot s^{-1}$, the larger clams did not saturate at any ambient light intensity (up to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Fig. 5). Since oxygen evolution measurements could not therefore be made at "P_{max}" for all clams, we chose 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ as the experimental irradiance because this is well above the saturation irradiance for isolated zooxanthellae (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Fig. 6), and in the top of the range experienced by the clams *in situ*. In order to determine the relation between oxygen production and the size of the clam, oxygen flux rates in the dark and at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ were measured for a wide size range of clams. Photo-

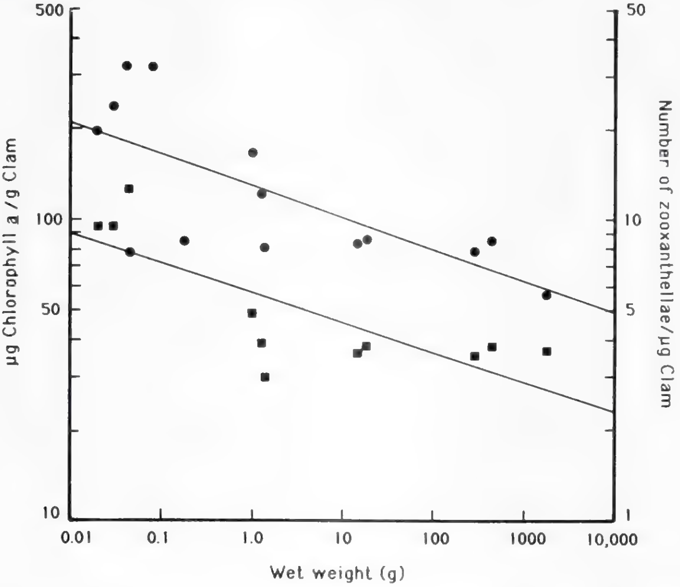

FIGURE 1. Number of zooxanthellae and amount of chlorophyl *a* per gram (wet weight) of *T. gigas* as a function of the wet weight of the clam (without shell). Squares represent the number of zooxanthellae per μg clam ($y = 5.67x^{-0.0966}$, $n = 11$, $r = 0.77$, $P < 0.01$). Circles represent the μg Chl. *a* per gram clam ($y = 127x^{-0.1035}$, $n = 14$, $r = 0.69$, $P < 0.01$).

synthetic oxygen production rates of the zooxanthellae *in situ* were inversely related to the size of the clam (Fig. 2).

The decrease in number of zooxanthellae per gram of clam (Fig. 1) and in *in situ* productivity of those zooxanthellae with increasing size of the clam (Fig. 2) results in a strong inverse relation between oxygen production rate and size of the clams (Fig. 3). The rate of respiratory oxygen consumption is also inversely related to the size of the clams (Fig. 3).

The relation between photosynthetic oxygen evolution and irradiance (P-I) was determined for 7 clams ranging from 1.15 to 23.5 cm in shell length (0.02–290 g wet weight). Four representative curves are presented in Figure 5. Both alpha/g (the slope of the initial light limited portion of the P-I curve) and "P_{max}" (assumed maximum photosynthetic rate) decrease with increasing size of the clam. The smallest clams showed light saturation of photosynthesis by 600 $\mu E \cdot m^{-2} \cdot s^{-1}$, but the larger clams were not saturated even at 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$.

P-I relations were also determined for zooxanthellae isolated from the mantles of three different clams. One of these curves, together with the P-I curve for the intact clam from which the zooxanthellae were isolated, is shown in Figure 6. The freshly isolated zooxanthellae did not show any evidence of photoinhibition up to maximum ambient illumination (2000 $\mu E \cdot m^{-2} \cdot s^{-1}$), although the P_{max} for freshly isolated zooxanthellae was well below that of the intact clam. The dark respiration rates averaged 8.5% (± 1.8, $n = 9$) of P_{max}. The alpha values for freshly isolated zooxanthellae ranged from 0.0019 to 0.0038 ($\mu MO_2 \cdot \mu gChla^{-1} \cdot \mu E^{-1} \cdot m^2$), but were not correlated with the size of the clam from which the zooxanthellae were isolated. P_{max} (photosynthetic rate at 600 $\mu E \cdot m^{-2} \cdot s^{-1}$) was measured for algae isolated from eleven different clams. The rates varied over a two-fold range when calculated on the basis of either chlorophyll

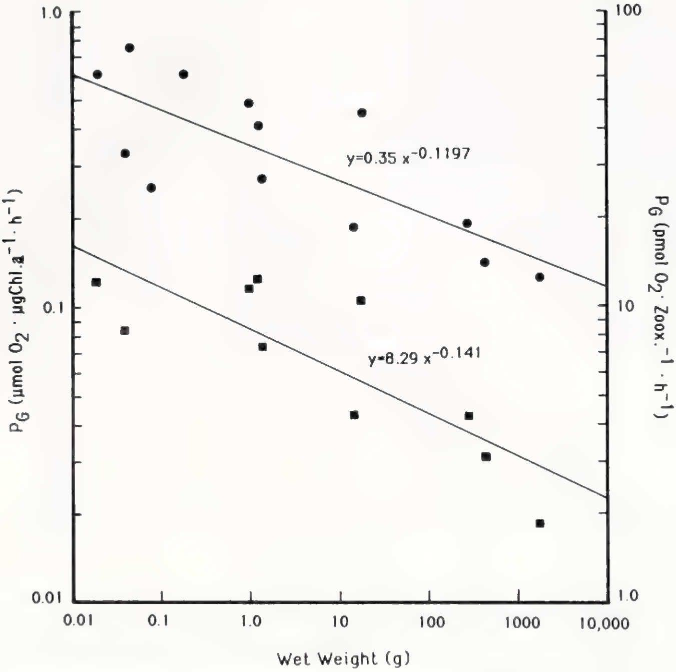

FIGURE 2. Gross photosynthetic oxygen production (P^G) in *T. gigas* at 1000 μE · m^{-2} · s^{-1} as a function of the wet weight of the clam. Squares represent pmol oxygen produced per zooxanthella per hour ($y = 8.29x^{-0.141}$, n = 10, r = 0.82, $P < 0.01$). Circles represent μmol oxygen produced per μg Chl. *a* per hour ($y = 0.35x^{-0.1197}$, n = 13, r = 0.78, $P < 0.01$).

a or algal cell number. The values were independent of the size of the clam from which the zooxanthellae were isolated (Fig. 7).

Using these data, an envelope enclosing all possible P-I relations for the freshly isolated zooxanthellae was constructed and then superimposed on P-I curves of the same four clams shown in Figure 5, with oxygen production rates presented per μg Chl. *a* so that the direct comparison can be made between the performance of intact clams and freshly isolated zooxanthellae (Fig. 8). The alpha values of the smallest clams are within the range of values of the freshly isolated zooxanthellae. However, the alpha values of the larger clams decrease progressively as the size of the clams increases. The P_{max} values of all but the largest clams are higher than the highest P_{max} measured for freshly isolated algae.

The ratio of gross oxygen production to consumption over a 24-hour period (P/R) and the percent contribution of carbon translocated from the zooxanthellae to animal respiration (CZAR) was calculated for each clam (or group of clams), using a variety of assumptions. The daily P/R assumed 10 hours of sunlight at either 500 or 1000 μE · m^{-2} · s^{-1} and 24 hours of respiration at the same rate as that measured in the dark. CZAR was calculated using the methods of Muscatine *et al.* (1981). The theoretical equation for this calculation is: CZAR = (P_{Gross}Zoox. for 24 h) (%translocation) ÷ (R Animal for 24 h). The specific assumptions we made for our calculations of CZAR are the following; algal biomass and the algal contribution to the overall respiratory rate of the clam is approximately 5% (Trench *et al.*, 1981). Daily photo-

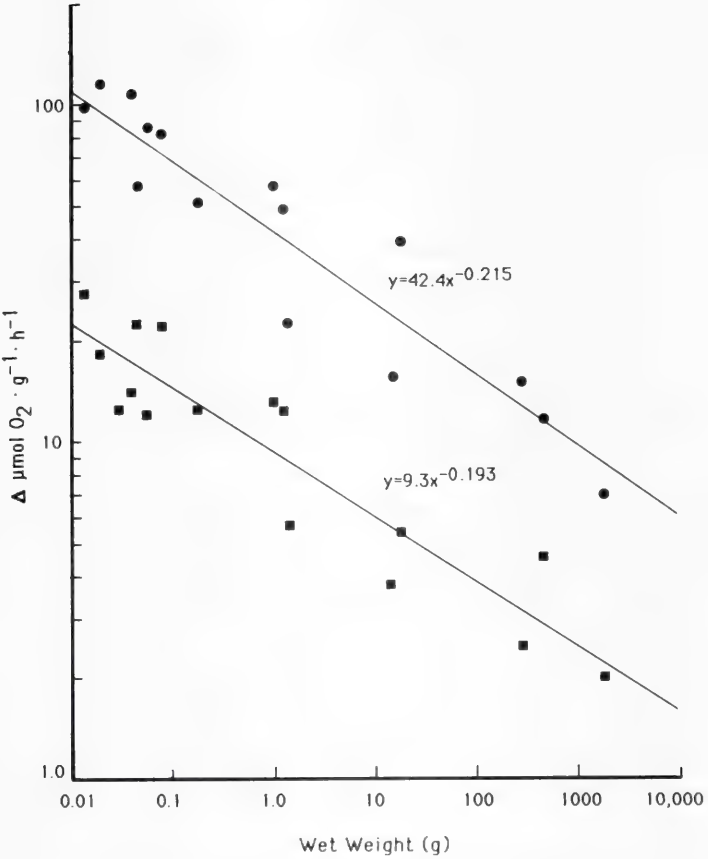

FIGURE 3. Oxygen evolution (P_g) at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ and respiration (R) per gram clam per hour as a function of the wet weight of the clam. Circles represent the gross photosynthetic rate ($y = 42.4x^{-0.215}$, $n = 15$, $r = 0.94$, $P < 0.01$). Squares represent the respiration rate ($y = 9.3x^{-0.193}$, $n = 16$, $r = 0.93$, $P < 0.01$).

synthesis is approximately equal to 10 hours of sunlight at an irradiance of 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$. The dark respiration rate is equal to the light respiration rate. Both the PQ and RQ are approximately equal to 1.0. We calculated CZAR twice assuming two different values for translocation: 40% (Trench *et al.*, 1981) and 95% (Muscatine *et al.*, 1984). For an in-depth discussion of these and other assumptions inherent in these calculations, see: Muscatine and Porter (1977); McCloskey *et al.* (1978); Muscatine (1980); and Trench *et al.* (1981); Muscatine *et al.* (1984).

Daily ratios of photosynthesis:respiration (and therefore CZAR) at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ were not correlated with the size of the clam (Fig. 4a), but at 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ there was an inverse relation between P/R and increasing size of the clams (Fig. 4b). Daily P/R and CZAR values are presented in Table I. Assuming 10 hours of sunlight at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$, P/R values ranged from 1.1 to 3.2 and averaged 1.9. Assuming either 40% or 95% translocation the calculated values of CZAR range from a minimum of 44% to a maximum of 318%.

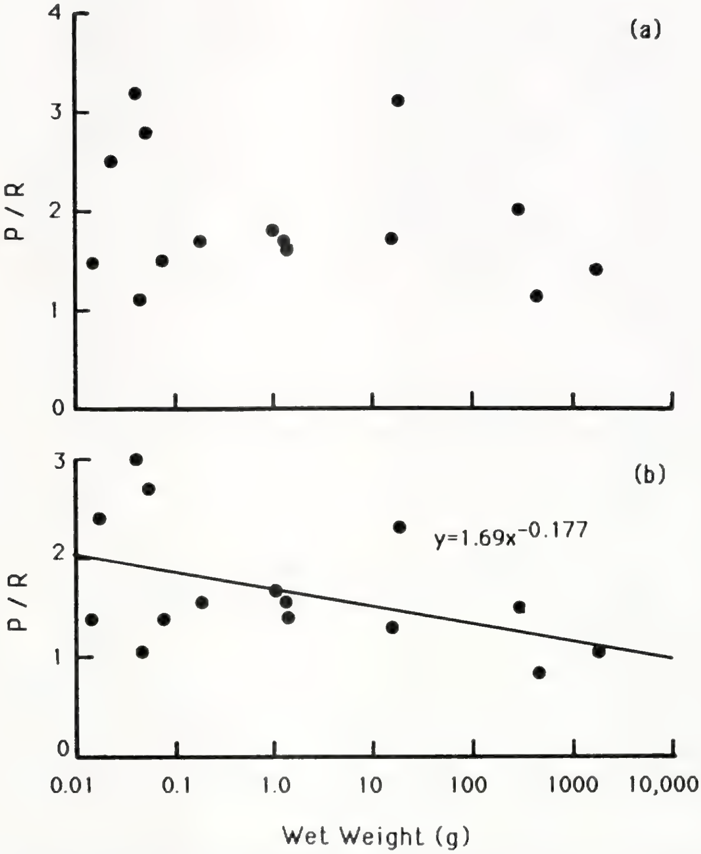

FIGURE 4. The ratio of gross photosynthesis to respiration (P/R) as a function of the wet weight of the clam at two different levels of irradiance. (a) P/R as a function of wet weight at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (n = 15, r = 0.27). (b) P/R as a function of wet weight at 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (y = −0.177 log x + 1.69, n = 15, r = 0.468, *P* = 0.1).

DISCUSSION

The total number of zooxanthellae in *Tridacna gigas* increased with the size of the clam, however the number of zooxanthellae per gram wet tissue decreased with increasing clam size (Fig. 1). Intuitively, one might expect that this relation occurs because the weight of the clam increases as a cube function while the area of the mantle increases as a square function. If this were true, then the allometric equation relating the total number of zooxanthellae (or total Chl. *a*) to the weight of the clam would have an exponent of 0.67. However, the allometric equations relating these parameters are;

$$\# \text{ of Zoox.} \cdot 10^{-6} = 5.623\ (\text{weight})^{0.9} \qquad (r = 0.996,\ n = 11,\ P < 0.01)$$

and

$$\mu g \text{ Chl. } a = 128.8\ (\text{weight})^{0.89} \qquad (r = 0.996,\ n = 14,\ P < 0.01)$$

The exponents are considerably greater than the expected 0.67, which is probably a reflection of two facts. First, not all of the zooxanthellae are found in the "mantle"

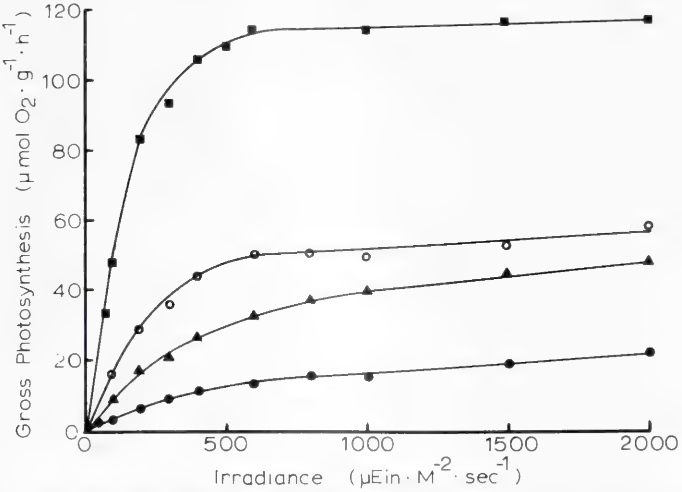

FIGURE 5. Gross photosynthesis as a function of irradiance (P-I curves) for four different size classes of clams. Each data point represents the rate of oxygen evolution over at least a ten-minute period measured when the oxygen level in the respirometry chamber was between 80 and 100% of air saturation; closed circles—wet weight = 1700 g: closed triangles—wet weight = 18.2 g: open circles—wet weight = 1.25 g: closed squares—six clams with an average wet weight of 0.014 g.

of the clams. In this study an average of 13% (±6.2%, n = 8) of the Chl. *a* was found in the "remains" portion of the clam (no correlation was found between the size of the clam and the distribution of the Chl. *a* in the various tissues). Second, as the clam increases in size, the "mantle" increases in thickness. Thus, there is a third dimensional component in the amount of "mantle" tissue, where 87% of the zooxanthellae reside.

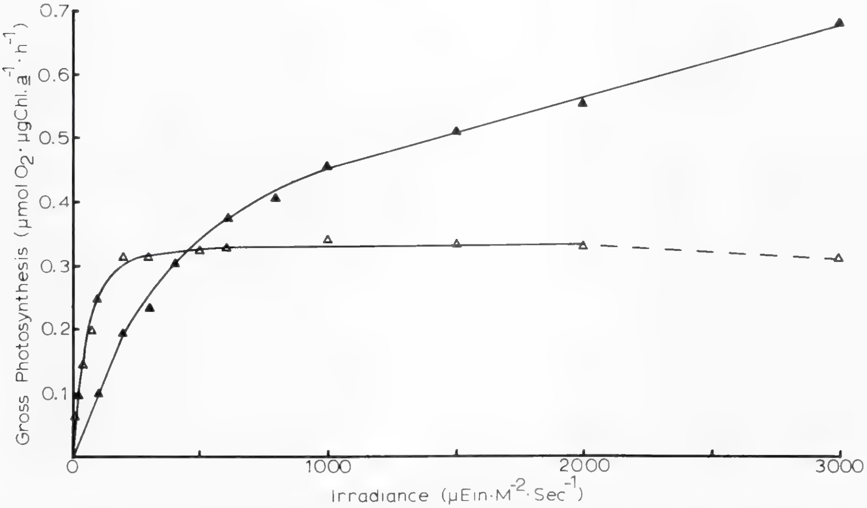

FIGURE 6. Photosynthesis as a function of irradiance (P-I curves) for the 18.2 g clam and zooxanthellae freshly isolated from it, calculated on the basis of Chl. *a* content: open triangles represent the freshly isolated zooxanthelle; closed triangles represent the intact clam.

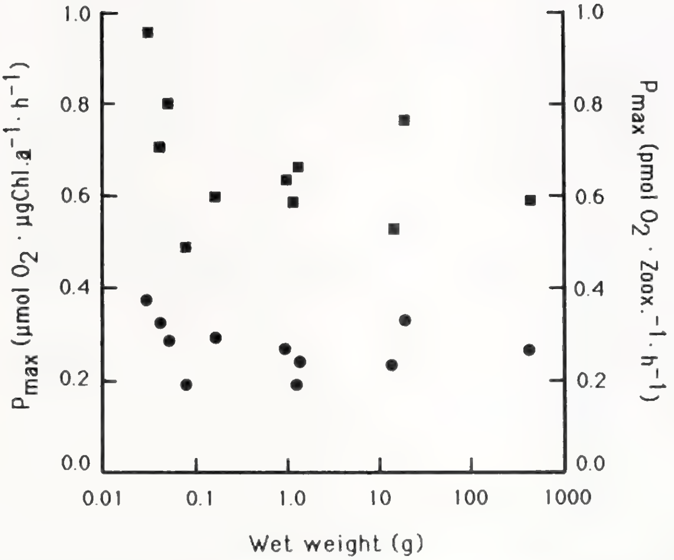

FIGURE 7. Gross photosynthetic oxygen evolution by freshly isolated zooxanthellae at a saturating light intensity of 600 μE · m^{-2} · s^{-1} (P_{max}) as a function of the wet weight of the clam from which the zooxanthellae were isolated. Closed squares represent P_{max} in pmol oxygen produced per zooxanthella per hour; closed circles represent P_{max} in μmol oxygen produced per μg Chl. *a* per hour.

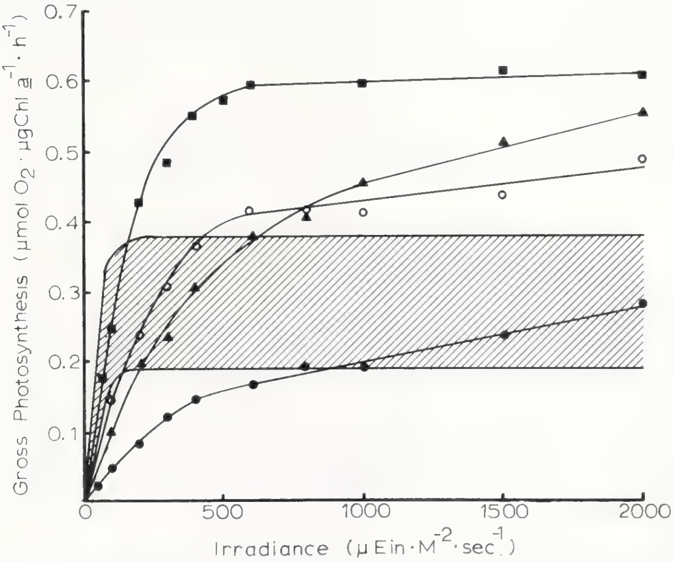

FIGURE 8. Gross photosynthesis as a function of irradiance (P-I curves) for four different size classes of clams and freshly isolated zooxanthellae. Note these are the same clams depicted in Figure 6 but P_g is expressed in μmol oxygen · μg Chl. *a* $^{-1}$ · h^{-1} instead of μmol oxygen · g^{-1} · h^{-1}: closed circles—wet weight = 1700 g: closed triangles—wet weight = 18.2 g: open circles—wet weight = 1.25 g: closed squares—six clams with an average wet weight of 0.014 g. The hatched area represents a composite of all P-I curves obtained for zooxanthellae freshly isolated from *T. gigas*.

The effects of the decreasing number of the zooxanthellae per gram of clam and the increased shading of those zooxanthellae with increasing clam size (discussed below) are reflected in the strong inverse relation between decreasing productivity (O_2 evolved/g clam/h) at a given illumination and increasing size of the clam (Fig. 3).

Light impinging on the surface of the mantle of a clam must penetrate increasingly thicker tissue as the size of the clam increases, before reaching all of the zooxanthellae, which are themselves stacked (Trench *et al.*, 1981). Evidence of increased shading of the zooxanthellae in the "mantles" of the larger clams comes from two different series of experiments. In one series of experiments gross photosynthesis at 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ was measured for 13 clams. A decrease in P_G was correlated with increasing clam size, even when P_G was expressed on the basis of Chl. *a* or number of zooxanthellae (Fig. 2). In another series of experiments P-I curves were generated for seven clams. The decrease in alpha and "P_{max}" with increasing clam size is indicative of the increased shading of the zooxanthellae in the larger clams (Table II, Fig. 8). Only the smallest clams show saturation of photosynthesis at ambient light intensities whereas the freshly isolated zooxanthellae saturate at 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Figs. 5, 6) (also Scott and Jitts, 1977; Trench and Fisher, 1983). The lack of saturation of photosynthesis in the larger clams can be explained by the observation that as light intensity is increased, saturating levels of irradiance are reaching deeper into the mantle tissues. In order to put a value on this shading phenomenon, one can compare the P-I curves of intact clams to P-I curves of freshly isolated zooxanthellae. Superimposed on the P-I curves of the four clams in Figure 8 is a hatched area which represents all of the possible P-I curves obtained for freshly isolated zooxanthellae. When we speak of the shading of the zooxanthellae in a certain sized clam, what we mean is the average amount of shading throughout all of the zooxanthella-containing tissues. For example, in a large clam the zooxanthellae on the surface of the mantle may be light-saturated at 400 $\mu E \cdot m^{-2} \cdot s^{-1}$, while those deep in the mantle are perceiving only a small fraction of the incident light. Because of this, it is necessary to compare the alpha values of the P-I curves; this is the only portion of the curves where all of the zooxanthellae are light limited. We therefore propose an equation to estimate the degree of shading in a clam:

$$\% \text{ shading} = (1 - \text{alpha of intact clam})(100) \div (\text{alpha of freshly isolated Zoox.})$$

TABLE II

Shading of Tridacna gigas *zooxanthellae*

Wet weight (g)	Shell length (cm)	Alpha/Chl. *a*	% Shaded Min	% Shaded Max
0.02	1.15	0.0025	0	34
0.041	1.55	0.0018	5	53
0.052	1.54	0.0018	5	53
0.84	4.07	0.002	0	47
1.25	4.82	0.0015	21	60
18.2	10.3	0.0010	47	74
290	23.5	0.0005	74	87

Alpha/Chl. *a* for freshly isolated zooxanthellae ranged from 0.0019 to 0.0038 and the minimum and maximum % shading experienced by zooxanthellae in the clam was calculated by using these values in the following equation:

$$\% \text{ shaded} = [1 - (\text{alpha clam}) \div (\text{alpha zooxanthellae})](100)$$

In this equation the ratio of alpha values is equal to the ratio of photosynthetic rates at a given limiting light intensity. The range of alpha values calculated for the seven clams for which P-I curves were generated are shown in Table II. In the smaller clams the mantle is relatively thin and shading would not significantly reduce the maximum photosynthetic rate the zooxanthellae can achieve. The zooxanthellae in the larger clams are estimated to receive, on the average, only 25% of the incident light. Muller-Parker (1984) found no significant shading of zooxanthellae *in situ* in *Aiptasia pulchella.* This is not surprising because, as the author pointed out, *Aiptasia* is a very small, unpigmented anemone. On the other hand, Crossland and Barnes (1977) determined that the algae in the coral *Acropora acuminata* were significantly shaded *in situ.* Application of the % shading equation to the data of Crossland and Barnes (1977), indicates that on the average about 20% of the incident light reached the zooxanthellae *in situ.* This low value is probably a reflection of both the position of the algae in the coral's tissue and the geometry of the staghorn coral. The zooxanthellae on the underside of a coral branch could be shaded by the coral skeleton itself.

The P_{max} of freshly isolated zooxanthellae is significantly lower than the P_{max} of all but the largest clam measured (Fig. 6, 8). This phenomenon, while difficult to interpret, could be attributable to a number of causes: (1) physical damage to some of the zooxanthellae during the isolation procedure. This damage might not be discernible at the level of light microscopy, and could result in partial photosynthetic inactivation; (2) zooxanthellae in isolation could be producing superoxide anions (O_2^-) and therefore hydrogen peroxide (H_2O_2), which is not detectable with an oxygen electrode. The deleterious effects of O_2 and H_2O_2 would normally be controlled by superoxide dismutase and catylase present in host tissue (Dykens and Shick, 1982; Dykens, 1984; and E. M. Tytler, pers. comm.); (3) the host milieu could stimulate high rates of photosynthesis in zooxanthellae. This could be due to *in situ* pH or to some other "factor" in the host (Trench, 1971b, Deane and O'Brien, 1980). None of these possibilities would increase the alpha values for freshly isolated zooxanthellae. The only effect on alpha that any of these possibilities would have is to lower the alpha value for freshly isolated zooxanthellae which would make our estimates of shading conservative minimum values.

When comparing Figures 5 and 8 it is apparent that the trend of decreasing alpha and P_{max} with increasing clam size becomes somewhat blurred when photosynthesis is expressed on the basis of chlorophyll *a* (Fig. 5). This can easily be accounted for by the well known effects of photoadaptation on Chl. *a* content in zooxanthellae (Zvalinskii *et al.*, 1980; Dustan, 1982; Chang *et al.*, 1983). In this study the variation in chlorophyll *a* content per zooxanthella was not correlated with the size of the host clam. Chlorophyll *a* per cell was not determined for the smallest clams because the small amount of material present in those clams did not allow that determination as well as assaying total Chl. *a* per clam. In the larger clams the zooxanthellae in the hemal sinuses of the "mantle" are shaded to different degrees depending on their position in the mantle. Therefore, any changes in Chl. *a* content of the zooxanthellae deep in the mantle are obscured by the presence of "surface" algal cells in the same extract.

The relation between photosynthesis and size discussed above would yield a similar relation between P/R and size were it not for the fact that respiration rate also decreases with increasing body size (Fig. 3). The exponent of the equation relating body weight to respiration falls between 0 and 1 for most organisms (R is expressed as total oxygen consumed per unit time) and is a function of the allometric increase in metabolism with growth (Zeuthen, 1953; Schmidt-Nielsen, 1974). This value ranges from 0.595 to 0.93 for *Mytilus edulis* (Kruger, 1960; Read, 1962). Bayne *et al.* (1976) averaged the values obtained in a large number of studies on marine mussels and found a value of 0.71. The value for *T. gigas* of 0.81 found in this study ($R = 8.0 W_{0.81}$) is higher than this average, but well within the range of values found for *Mytilus*, and while difficult to interpret may reflect a difference in temperature, feeding, season, or stage in life history of the animals (Zeuthen, 1953; Kruger, 1960; Kuenzler, 1961; Widdows, 1978; and Walsh and Somero, 1981; *cf.* Read, 1962; Ansell, 1973).

The calculated values of daily P/R and especially of CZAR vary greatly depending upon the assumptions one makes in the calculations (Table I). Nevertheless, it is clear that the zooxanthellae do play a very significant role in the nutrition of *T. gigas* and in fact may be able to supply as much as 100% of the respiratory carbon requirements of the clam. At high light intensities ($\geq 1000\ \mu E \cdot m^{-2} \cdot s^{-1}$) the variation in the P/R for the intact symbiosis shows no strong correlation with the size of the clam (Table I, Fig. 4a). However, if one assumes an average daily illumination of 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ such as the clam would experience on a cloudy day or in deeper water, an inverse relation between P/R and clam size is evident (Table I, Fig. 4b). The difference between Figures 4a and b can be understood by inspecting the P-I curves for the four different sizes of clams shown in Figure 5. Lowering the light intensity from 1000 to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ lowers the photosynthetic rate of the smallest clams by only 4%, while it results in a 25% reduction of the photosynthetic rate of the two largest clams. This reduction is due to the increased shading in the larger clams as described above.

These physiological parameters of *Tridacna gigas* are of special import to a mariculture operation. Juvenile clams can be grown optimally at light intensities that are significantly below ambient. This should help reduce algal fouling of the growth tanks. On the other hand, since zooxanthellae freshly isolated from *T. gigas* showed no evidence of photoinhibition at even the highest ambient light intensities (2000 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Fig. 6), and since the photosynthetic rates of the larger clams are not saturated at ambient levels of irradiance (Fig. 5), the higher the intensity at which one grows the larger clams, the better.

ACKNOWLEDGMENTS

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5-HYDROXYTRYPTAMINE MEDIATES RELEASE OF MOLT-INHIBITING HORMONE ACTIVITY FROM ISOLATED CRAB EYESTALK GANGLIA

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ABSTRACT

Neurosecretory cells in crustacean eyestalk ganglia produce a putative molt-inhibiting hormone (MIH) which directly suppresses production of the steroid molting hormone, ecdysone, by the peripheral Y-organs. Neurotransmitter mediation of MIH release from isolated eyestalk ganglia of the crab, *Cancer antennarius,* was explored using an MIH bioassay based upon *in vitro* inhibition of Y-organ ecdysteroid production by eyestalk ganglion-conditioned saline. The conditioned saline (0.01–1.0 eyestalk equivalent) inhibited Y-organ ecdysteroid production dose-dependently and reversibly, and the effect of the saline was specific as to conditioning tissue. Isolated ganglia released a significant portion of their MIH activity in 2-h incubations, but also retained a significant portion. 5-hydroxytryptamine (5-HT) enhanced MIH release at concentrations of 10^{-10} *M* to 10^{-6} *M*. Acetylcholine, dopamine, octopamine, norepinephrine, or gamma-aminobutyric acid (10^{-7} *M*–10^{-6} *M*) did not alter basal MIH release. The 5-HT precursor, 5-hydroxytryptophan (10^{-7} *M*), stimulated MIH release, while the tryptophan hydroxylase inhibitor, p-chlorophenylalanine (10^{-6} *M*), and the 5-HT receptor antagonist, cyproheptadine (10^{-7} *M*), inhibited MIH release to below basal levels. Apparently, 5-HT neurons provide excitatory input to MIH-containing neurosecretory cells.

INTRODUCTION

In decapod crustaceans, the steroidogenic Y-organs are the source of the molting hormone, ecdysone (Chang *et al.,* 1976; Chang and O'Connor, 1977). Eyestalk extirpation and implantation studies (Carlisle, 1957; Gersch *et al.,* 1977) and *in vitro* suppression of steroidogenesis in Y-organ tissue segments and dispersed cells by eyestalk extracts (Mattson and Spaziani, 1985a, b; Watson and Spaziani, 1985) indicate that an eyestalk factor (MIH) directly inhibits Y-organs. MIH originates in eyestalk neurosecretory cells (X-organs) with axonal endings that form a neurohemal organ, the sinus gland (SG) (see reviews by Kleinholz, 1976; Cooke and Sullivan, 1982).

Evidence exists that suggests a synaptic basis for control of neurohormone release from the X-organ-SG complex. Isolated X-organ-SGs change electrical activity in response to 5-hydroxytryptamine (5HT) and gamma-aminobutyric acid (GABA) (Nagano and Cooke, 1981). In addition, studies by Fingerman and co-workers on the release of chromatophorotropic hormones from eyestalks *in vivo* and *in vitro* in *Uca pugilator* suggest that 5HT, dopamine (DA), and norepinephrine (NE) induce release of a red pigment-dispersing hormone, red and black pigment-concentrating hormones,

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Abbreviations: ACH, acetylcholine; CPH, cyproheptadine; DA, dopamine; GCS, eyestalk ganglion-conditioned saline; ESE, eyestalk equivalent; GABA, gamma-aminoisobutyric acid; 5-HT, 5-hydroxytryptamine; 5HTP, 5-hydroxytryptophan; MIH, molt-inhibiting hormone; NE, norepinephrine; OA, octopamine; PCPA, p-chlorophenylalanine; RIA, radioimmunoassay; SG, sinus gland.

and black pigment-dispersing hormones, respectively (Fingerman and Fingerman, 1977a; Fingerman *et al.*, 1981; Quackenbush and Fingerman, 1984). Higher-order neurons with specific neurotransmitter phenotypes apparently control release of specific peptides from neurosecretory cells in mammalian pituitary (Mathison, 1981; McCann, 1981) and insect brain and corpus cardiacum (Samaranoyaka, 1976; Orchard *et al.*, 1983).

We reported recently a formal bioassay for MIH based on suppression by eyestalk extracts of ecdysteroid production in isolated Y-organ quarters of the crab *Cancer antennarius* (Mattson and Spaziani, 1985a). Soumoff and O'Connor (1982) showed that isolated whole Y-organs are inhibited in a dose-dependent manner by SG-conditioned saline from *Pachygrapsus crassipes*. The present study combines the MIH bioassay and the conditioned medium technique to measure release of MIH activity from isolated eyestalk whole ganglia exposed to neurotransmitters and agents that alter transmitter synthesis or receptor action. The experiments provide evidence that transmitter-specific higher-order neurons intrinsic to eyestalk ganglia supply excitatory input to MIH-containing neurosecretory cells.

MATERIALS AND METHODS

Female rock crabs, *Cancer antennarius* Stimpson, were obtained from Pacific Biomarine (Venice, CA) and Marinus, Inc. (Westchester, CA), and were maintained individually in compartments of water tables with constantly circulating, charcoal-filtered reconstituted sea water at 16–17°C. Crabs were fed fish three times weekly and were allowed to acclimate to their environment for at least two weeks prior to experimentation (Mattson and Spaziani, 1985c).

Eyestalks were cleaned with alcohol swabs, removed by cutting connective tissue and the optic nerve at the base, and transferred to sterile Pantin's saline (Pantin, 1934). The protruding stump of the optic nerve was grasped with a blunt forceps and the entire complex of optic ganglia (including the X-organ-sinus gland complex) was removed from the exoskeleton, freed of adhering connective and ommatidial tissue, and placed in saline (1 ganglia/100 μl saline) without (control) or with given concentrations of neurotransmitters or drugs. Contralateral ganglia from individual animals were randomly assigned to control and treatment plates to control for possible effects of interanimal variabilities in MIH neurosecretory activity. Incubations were at 20°C in an atmosphere of 50% oxygen/50% air with rotary shaking at 60 rpm for 2 h unless otherwise stated. In one experiment individual sinus glands were isolated by dissection and incubated under the above conditions. At the end of incubations the conditioned saline was removed, immersed in a boiling water bath for 2 min, and centrifuged at $1000 \times g$ for 10 min. The supernatant was removed, adjusted to the initial incubation volume with water, and stored frozen. 5HT, DA, NE, acetylcholine (ACH), octopamine (OA), GABA, 5-hydroxytryptophan (5HTP), p-chlorophenylalanine (PCPA), cyproheptadine (CPH), and reserpine were obtained from Sigma Chemical Co. (St. Louis, MO).

For the assay of MIH activity, activated Y-organs from 48 h de-eyestalked crabs were removed, quartered, and incubated for 24 h under conditions supporting maximal and sustained ecdysteroid production as previously described (Mattson and Spaziani, 1985a). Under these conditions Y-organ quarters produced between 90 and 130 pg of ecdysteroids/μg protein/24 h in all experiments in the present study. To Y-organ quarters incubated in 500 μl of medium (Medium 199 adjusted to crab serum osmolarity and supplemented 10% with fetal calf serum, and buffered with 2.0 g/l HEPES) was added 100 μl of saline (control) or of eyestalk ganglia-conditioned saline (GCS), SG-conditioned saline, or saline containing neurotransmitters or drugs (controls for possible direct action of these agents on Y-organ ecdysteroid production). When consecutive incubations (with intervening medium changes) were done, Y-organ tissue was washed twice (10 min/wash) with 500 μl of medium between incubations.

At the end of incubations, medium was removed and stored at 4°C for ecdysteroid RIA. At the end of final incubations Y-organ tissue was processed for protein quantification. Procedures for ecdysteroid RIA and preparation of tissue for protein assay have been previously described (Mattson and Spaziani, 1985a). Protein was quantified by the Bradford (1976) method using BSA as standard. Ecdysone antiserum (antibody H21B; Horn *et al.*, 1976; a gift from W. E. Bollenbacher, Dept. of Zoology, Univ. of North Carolina, Chapel Hill), tritium-labeled ecdysone (specific activity 60 mCi/mmol; New England Nuclear, Bedford, MA), and ecdysone standards (Research Plus, Bayonne, NJ) were used for the ecdysteroid RIA. As Y-organs produce more than one ecdysteroid with affinity for the antiserum used (Watson and Spaziani, 1985a) values represent ecdysone equivalents. Intra- and interassay variabilities were 40 and 80 pg, respectively at an ecdysone dose of 1 ng. Conditioned saline, neurotransmitters, and drugs used did not interfere with ecdysteroid RIA.

MIH activity in GCS from neurotransmitter- or drug-treated ganglia was calculated as the ratio of 24 h ecdysteroid production of Y-organ quarters exposed to control GCS divided by those exposed to GCS from treated ganglia, ×100. Control GCS from 2-h incubations inhibited Y-organ ecdysteroid production to 40–60% of Y-organ control levels in all experiments. Statistical comparisons were done using Student's *t*-test.

RESULTS

Time course of release of MIH activity from isolated eyestalk ganglia and sinus glands

We previously showed that ecdysteroid production by Y-organ quarters is inhibited dose-dependently by MIH activity in eyestalk extracts (Mattson and Spaziani, 1985a). Similar results were obtained in a dose-response study with GCS (Fig. 1). MIH activity in GCS inhibited 24-h ecdysteroid production by Y-organ quarters dose-dependently at concentrations of 0.01 to 1.0 eyestalk equivalents (ESE). Inhibition to less than 60% of control levels occurred at a GCS dose of 1 ESE; half maximal inhibition was obtained with 0.01–0.1 ESE (Fig. 1). As Soumoff and O'Connor (1982) found that isolated sinus glands from *Pachygrapsus crassipes* released a maximal amount of MIH activity within 1 h, we compared levels of MIH activity (as measured by degree of inhibition of Y-organ ecdysteroid production) released over time from isolated sinus glands and entire eyestalk ganglia complexes. This experiment was designed to determine an incubation time period which would allow release of MIH, but also the retention of measurable amounts of MIH activity in ganglia. Figure 2 shows that 1 eyestalk equivalent (ESE) of GCS from 2-h and 8-h incubations significantly inhibited Y-organ ecdysteroid production to 53% and 31% of control levels, respectively. MIH activity in 2-h GCS was intermediate to, and significantly different from, activity in 30-min and 8-h GCS. One ESE of SG-conditioned saline from 30-min, 2-h, and 8-h incubations significantly suppressed Y-organ activity to near 30% of control levels. MIH activity released from SGs at all three incubation times was significantly greater than activity released from ganglia incubated for 30 min and 2 h, but was similar to MIH activity in 8-h GCS. As intermediate amounts of MIH activity were released from eyestalk ganglia incubated for 2 h, this incubation time was chosen for all subsequent experiments.

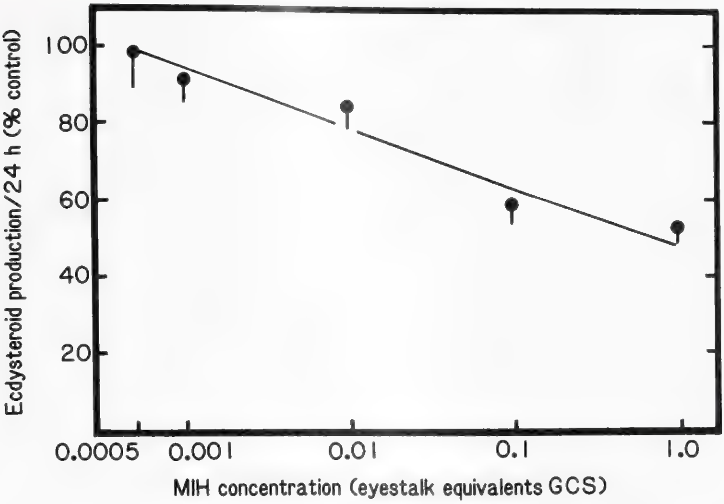

FIGURE 1. Dose-dependent inhibition of Y-organ activity by eyestalk ganglion-conditioned saline (GCS). Y-organ quarters were incubated 24 h with the stated concentrations (expressed as eyestalk equivalents ESE) of GCS and ecdysteroid production quantified by RIA. Points are each the mean and st. error of mean of 4 (0.0005 ESE) or 8 (0.001–1.0 ESE) incubations. Control Y-organs produced 128 ± 10 pg/μg gland protein of ecdysteroids. Inhibition was significant at GCS doses of 0.01 ESE or greater ($P < 0.05$–0.001).

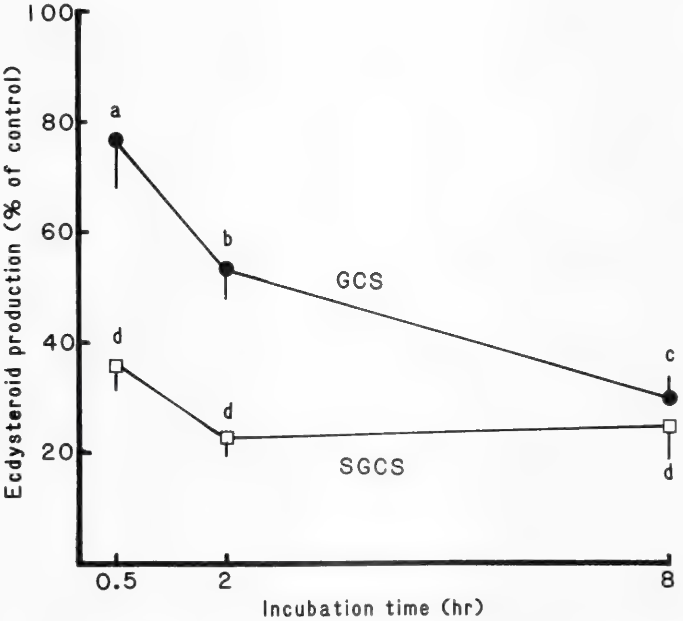

FIGURE 2. Effect of incubation time on release of MIH activity from isolated eyestalk ganglia and sinus glands. The tissues were incubated for the stated times in saline (1 ESE in 100 μl of saline), and MIH activity of the ganglion-conditioned (GCS)- and sinus gland-conditioned (SGCS)-salines determined by ability to inhibit ecdysteroid production by Y-organ quarters *in vitro* (24 h). Points are each the mean and st. error of mean of four incubations. Control Y-organ incubations produced 123 ± 18 pg of ecdysteroids/μg protein. a, $P < 0.001$ *vs* d; b, $P < 0.05$ *vs* a and c, $P < 0.01$ *vs* d; b, c and d, $P < 0.01$–0.001 *vs* control.

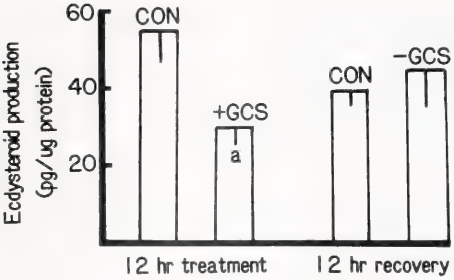

FIGURE 3. Reversibility of GCS-induced suppression of Y-organ function. Y-organ quarters incubated with (+GCS, 1 ESE) or without (CON) GCS for 12 h were washed twice (10 min/wash) with fresh medium and incubated an additional 12 h in the absence of GCS. Height of each bar and enclosed line is the mean and st. error of mean of four incubations. a, $P < 0.05$ *vs* CON.

Characterization of effects of GCS on Y-organ tissue ecdysteroid production

To determine whether GCS-induced inhibition of Y-organs was reversible, Y-organs were incubated for 12 h with 1 ESE of GCS, washed, and incubated an additional 12 h in the absence of GCS. Figure 3 indicates that Y-organ ecdysteroid production was significantly inhibited by GCS in the first 12-h incubation but was not significantly different from control ecdysteroid levels in the second 12-h incubation after GCS removal. To show that inhibition of Y-organ ecdysteroid production was specific for saline conditioned with eyestalk ganglia, saline conditioned with crab muscle or brain were tested for ability to alter Y-organ activity. Brain- and muscle-conditioned saline at concentrations equivalent to 1 ESE of GCS (equivalent wet weights/saline volume) did not significantly alter Y-organ ecdysteroid production in 24-h incubations, while GCS significantly suppressed ecdysteroid production to near 50% of control levels (Fig. 4).

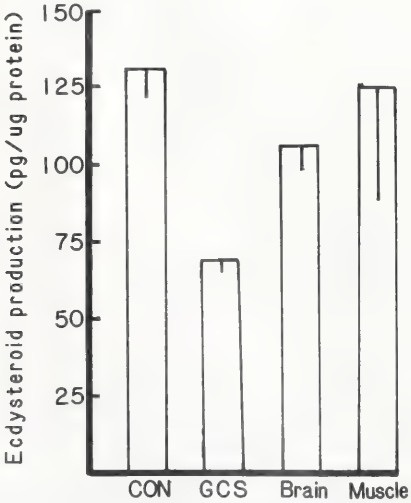

FIGURE 4. Tissue specificity of MIH activity released into saline. Ecdysteroid production of Y-organ quarters incubated for 24 h with 1 ESE of GCS or saline conditioned with crab brain or muscle (tissue weight/saline volume equal to 1 ESE) were compared. Bars and enclosed lines are the mean and st. error of mean of four incubations. GCS, $P < 0.01$ *vs* CON.

Effects of neurotransmitters on release of MIH activity from isolated eyestalk ganglia

Eyestalk ganglia were incubated for 2 h in saline containing a neurotransmitter, and MIH activity released into this saline was compared to that released into control saline. One ESE of GCS was used for control (basal) MIH release since it is clear from Figure 2 that the ganglion retained sufficient MIH activity in 2-h incubations to allow detection of changes in release of MIH activity caused by neurotransmitters. Control GCS inhibited Y-organs 40–60% in all experiments. Ganglia exposed to 10^{-7} *M* 5HT released significantly more MIH activity than control ganglia (Fig. 5). DA (10^{-6} *M*), NE (10^{-6} *M*), GABA (10^{-7} *M*), ACH, (10^{-7} *M*), and OA (10^{-6} *M*) had no significant effect on bioassayable MIH activity release from eyestalk ganglia. In control experiments in which neurotransmitters alone were added directly to cultured Y-organ tissue at concentrations equivalent to those in GCS it was found that DA (10^{-6} *M*) significantly inhibited ecdysteroid production to 40% of control levels (other neurotransmitters had no effect on Y-organ activity). However, when 10^{-6} *M* DA was incubated in saline alone for 2 h and the saline was carried through the usual boiling, centrifuging, freeze-thawing procedure for GCS (see Materials and Methods), this saline was ineffective in suppressing Y-organ ecdysteroid production. It was concluded, therefore, that DA does not affect MIH release.

Figure 6 shows that 5HT enhanced dose-dependently the release of MIH activity from isolated eyestalk ganglia. 5HT at 10^{-7} *M* caused maximal release of MIH activity; half maximal effect occurred with 5HT at approximately 10^{-10} *M*.

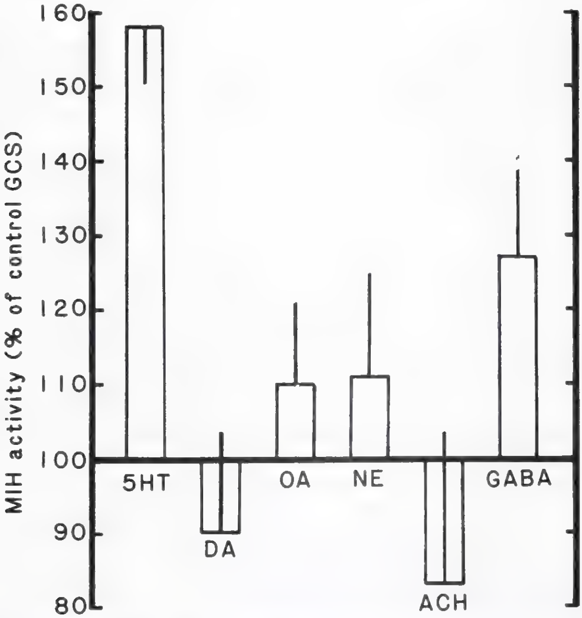

FIGURE 5. Effect of neurotransmitters on release of MIH activity from isolated eyestalk ganglia. MIH activity in 1 ESE of GCS from control (CON) or transmitter-treated ganglia in 2-h incubations were compared using the Y-organ bioassay for MIH. Control Y-organ tissue produced a mean of 95 pg of ecdysteroids/μg protein/24 h. Control GCS inhibited Y-organs 40–60% in all experiments. Bars and enclosed lines are the mean and st. error of mean of 8 (5HT) or 4 (other neurotransmitters) assays. Concentrations of neurotransmitters were 10^{-7} *M* for 5HT, gamma-aminobutyric acid (GABA), and acetylcholine (ACH); 10^{-6} *M* for dopamine (DA), norepinephrine (NE), and octopamine (OA). 5HT, $P < 0.05$ *vs* control (100%) GCS value.

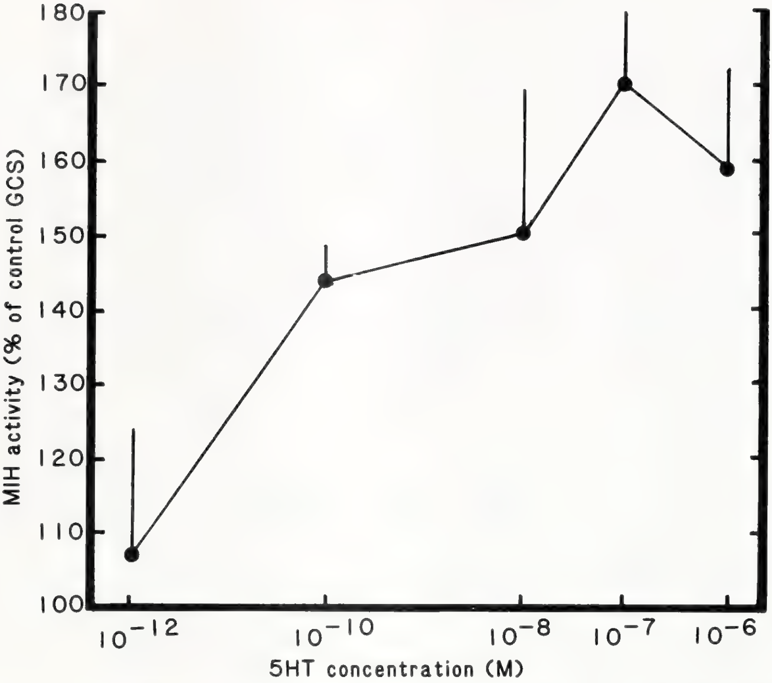

FIGURE 6. Dose dependency of 5HT-enhanced release of MIH activity from isolated eyestalk ganglia. MIH activities in 1 ESE of GCS from ganglia treated for 2 h with the given 5HT concentrations were measured using the Y-organ bioassay. Points are the mean and st. error of mean of 8 (10^{-7} *M*) or 4 (other 5HT doses) incubations. 5HT at doses of 10^{-10} *M* or greater significantly inhibited Y-organ ecdysteroid production ($P < 0.05$–0.02). Control Y-organ tissue produced 102 ± 20 pg of ecdysteroids/μg protein/24 h.

Effects of agents altering 5HT synthesis, release, or receptor activity on release of MIH activity

Isolated eyestalk ganglia exposed to the 5HT precursor 5HTP (10^{-7} *M*) released significantly more MIH activity (170%) than control ganglia (Fig. 7). GCS from ganglia treated with the tryptpophan hydroxylase inhibitor, PCPA (10^{-6} *M*) or the 5HT receptor antagonist, CPH (10^{-7} *M*), contained significantly less MIH activity (80%) than control GCS. Ganglia exposed to both 5HT and CPH (10^{-7} *M* each) showed no significant change in MIH release compared to control ganglia. The monoamine depletor, reserpine, at a concentration of 10^{-6} *M* did not have a significant effect on assayable MIH release.

DISCUSSION

A method for bioassay of MIH activity in crab eyestalk-conditioned saline (Figs. 1–4) has allowed assessment of neurotransmitter involvement in release of MIH activity and identification of 5HT as a stimulator of MIH release from the X-organ-SG neurosecretory complex in *Cancer antennarius* (Figs. 5–7). Release of maximal MIH activity from isolated SGs alone occurred within 30 min of incubation (Fig. 2), consistent with Soumoff and O'Connor's (1982) report that all detectable release of MIH activity from SGs of *Pachygrapsus crassipes* occurred in less than 1 h *in vitro.* In contrast, isolated eyestalk ganglia which contained intact X-organ-SG neurosecretory cells released MIH but also retained measurable MIH activity for several hours after

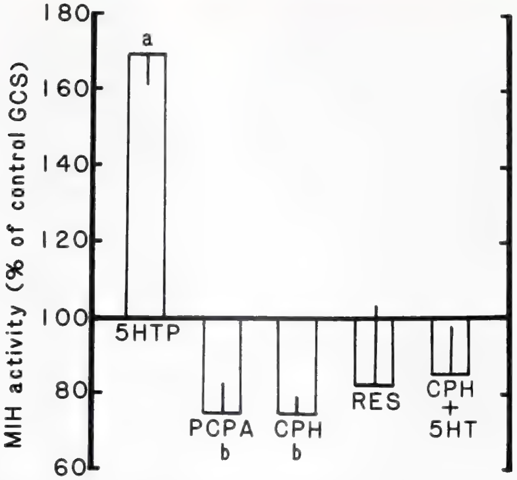

FIGURE 7. Effects of agents that alter 5HT synthesis, release, or receptor action on MIH activity released from isolated eyestalk ganglia. The Y-organ bioassay was used to compare MIH activities in 1 ESE of GCS from control (CON) or treated ganglia after 2 h incubations. Control Y-organ tissue produced a mean of 90 ± 13 pg of ecdysteroids/μg protein/24 h. Control GCS inhibited Y-organs 40–60% in all experiments. 5-hydroxytryptophan (5HTP) and cyproheptadine (CPH) concentration was 10^{-7} *M*; p-chlorophenylalanine (PCPA) and reserpine (RES), 10^{-6} *M*. For the experiment in which ganglia were exposed to both CPH and 5HT, ganglia were incubated in CPH-containing saline for 5 min prior to addition of 5HT. Bars and enclosed lines are the mean and st. error of mean of four assays. a, $P < 0.02$ *vs* control (100%) GCS value; b, $P < 0.05$ *vs* control.

removal to saline (Fig. 2). It is likely that severing X-organ axons during SG isolation induces MIH release. Doses of GCS and SG-conditioned saline capable of significantly inhibiting Y-organ activity (0.01 ESE, Fig. 1 and Soumoff and O'Connor, 1982) are an order of magnitude lower than doses of homogenized eyestalk extract required for inhibition (Mattson and Spaziani, 1985a; Watson and Spaziani, 1985); MIH activity appears to be lost in the extraction procedure, probably because of insufficient breakup of secretory granules. These results suggest that quantitative recovery of MIH may be obtained by collecting 8-h GCS, which contains MIH activity equal to that in SG-conditioned saline but does not require tedious dissection of SGs. The inhibitory effects of GCS on Y-organs were reversible and specific (Figs. 3, 4) consistent with previously reported effects of eyestalk extracts using the same bioassay culture system (Mattson and Spaziani, 1985a).

Within limits of sensitivity of our MIH bioassay, 5HT induces release of MIH activity from isolated eyestalk ganglia at concentrations of 10^{-10} *M* or greater, consistent with concentrations of neurotransmitters causing release of chromatophorotropic hormones from isolated eyestalks in *Uca pugilator* (Quackenbush and Fingerman, 1984). Our findings that the 5HT precursor, 5HTP, stimulates, while PCPA (an inhibitor of 5HT synthesis) or CPH (a 5HT receptor blocker) inhibits release of MIH activity (Fig. 7), suggest that neurons exist in eyestalk ganglia that provide 5HT input to MIH-containing cells. The effects of PCPA and CPH in lowering MIH release to below basal (control) levels suggest interference with endogenous 5HT. In addition, CPH blocked the stimulatory effect of exogenously applied 5HT, indicating a specific effect on 5HT receptors. Although a significant inhibition of release of MIH activity was not obtained with the monoamine depletor, reserpine, due to variability between ganglia, the mean level of MIH activity released by reserpine-treated ganglia was identical to levels released by PCPA-treated ganglia. Morphological studies indicate few or no synapses on X-organ somata or axonal terminals in SGs, with many synapses localized to neurites (Cooke and Sullivan, 1982).

It is likely, therefore, that 5HT receptors antagonized by CPH (Fig. 7) are localized to neurites. Other neurotransmitters tested in the present study were ineffectual in altering release of MIH activity. This suggests that if higher-order neurons control the 5HT neurons providing input to MIH-containing cells they are either located elsewhere in the CNS or they are also serotonergic; intrinsic 5HT neurons may also be directly responsive to visual inputs (*e.g.,* light, movement).

In the course of control experiments we found that DA applied directly to cultured Y-organ tissue caused significant inhibition of ecdysteroid production; the processing procedure for GCS (see Materials and Methods) abolished this DA activity. DA was likely oxidized by the incubation, boiling process. We are currently exploring the direct action of DA on Y-organ tissue and preliminary evidence suggests that DA (as is the case for MIH activity, Mattson and Spaziani, 1985b) acts through the cyclic AMP second messenger system (unpub. obs.).

Evidence suggests that release of specific neurotransmitters by neurons impinging on neurosecretory cells results in release of specific neurohormones. NE and DA stimulate release of leuteinizing hormone-releasing hormone (Negro-Vilar *et al.,* 1979), while cholinergic and beta-adrenergic stimulation induce vasopressin release from hypothalamic-hypophyseal cells in mammals. Neurosecretion is induced by 5HT in insects (Scharrer and Wurzelmann, 1978), and hyperglycemic hormone and erythrophore-concentrating hormone are released from crustacean X-organ-SG cells in response to 5HT and DA stimulation, respectively (Keller and Beyer, 1968; Fingerman and Fingerman, 1977a). However, it is apparent that selective activation of subgroups of neurons with a particular transmitter phenotype must occur in order for specific neurohormones to be released in response to specific environmental stimuli. In crustaceans, 5HT induces release from eyestalks of red pigment-dispersing hormone (Fingerman and Fingerman, 1977a), hyperglycemic hormone (Keller and Beyer, 1968), and MIH (present study). Combined data from several laboratories suggests that independent groups of 5HT neurons may respond to different (or similar) environmental stimuli. Thus red pigment-dispersing hormone release is maximal in daylight (Fingerman and Fingerman, 1977b), while hyperglycemic hormone release is maximal in darkness (Dean and Vernberg, 1965). In addition, both hyperglycemic hormone (Keller, 1983) and MIH (Mattson and Spaziani, 1985c) are apparently released in response to environmental stressors. Control of release from neurosecretory cells by neurons containing classical neurotransmitters thus appears complex and future immunohistochemical, biochemical, and electrophysiological experiments will be required to identify and quantify neurosecretory cells in crustaceans containing specific hormonal products, to assess possible processing of larger precursor molecules, and to elucidate neural inputs controlling release. In the case of MIH definitive studies in these areas require isolation and purification of MIH and anti-MIH antibody production.

ACKNOWLEDGMENTS

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A NOVEL FMRFamide-RELATED PEPTIDE IN *HELIX:* pQDPFLRFamide

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ABSTRACT

A novel FMRFamide-like peptide, purified from the ganglia of *Helix aspersa,* has the amino acid sequence: pyroglutamyl-aspartyl-prolyl-phenylalanyl-leucyl-arginyl-phenylalanine amide (pQDPFLRFamide). Synthetic pQDPFLRFamide was prepared; it is chromatographically and biologically indistinguishable from the natural peptide, confirming the sequence. pQDPFLRFamide is about a hundred times more potent than FMRFamide on the isolated *Helix* heart, but slightly less potent than FMRFamide on the *Busycon* radula protractor muscle. Since pQDPFLRFamide occurs in *Helix* blood at levels sufficient to excite the isolated *Helix* heart, it may act as a cardioregulatory hormone.

INTRODUCTION

The molluscan neuropeptide FMRFamide (phenylalanyl-methionyl-arginyl-phenylalanine amide) was originally isolated from the ganglia of a clam, *Macrocallista nimbosa* (Price and Greenberg, 1977a), but its occurrence is not restricted to this species, nor even to clams in general. Indeed, the ganglia of all molluscan species examined contain FMRFamide-like biological activity (Agarwal *et al.,* 1972). Moreover, FMRFamide itself appears to be ubiquitous in molluscs and, in some species [*e.g., Aplysia brasiliana* (Lehman *et al.,* 1984)], to account for all the FMRFamide-like activity present. In some gastropods, however, related peptides also occur and may even be the predominant forms (reviewed in Price, 1986).

Pulmonate ganglia, in particular, have activity not attributable to FMRFamide. In *Helix aspersa,* for example, the ganglia contain, not only FMRFamide, but also related peptides distinguishable by their chromatographic behavior and biological activity (Cottrell *et al.,* 1981; Price, 1982). Cottrell *et al.* (1981) found that partially purified FMRFamide from the ganglia of *Helix aspersa* has much more excitatory activity on the *Helix* heart than could be accounted for by FMRFamide itself, and Greenberg and Price (1980) used the heart of *Helix* and the radula protractor muscle of *Busycon* as a parallel bioassay to calculate this excess at about 600-fold.

Here we describe the purification of one of these peptides and the determination of its amino acid sequence as: pyroglutamyl-aspartyl-prolyl-phenylalanyl-leucyl-arginyl-phenylalanine amide (pQDPFLRFamide). We show that, although pQDPFLRFamide is somewhat less potent than FMRFamide on the radula protractor, it is about 100 times more potent than FMRFamide on the heart of *Helix.* Thus this heptapeptide can account for most of the excess cardioexcitatory activity found in *Helix* brain. Furthermore, we show that the blood levels of pQDPFLRFamide in *Helix* are sufficient to affect the heart, so it may be a cardioregulatory hormone.

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MATERIALS AND METHODS

Animals

Helix aspersa were collected in southern California and Scotland. Whelks (*Busycon contrarium*) were collected from the Gulf of Mexico south of Tallahassee, shipped to the Whitney Laboratory, and maintained there in natural seawater (31‰) at 23°C. Clams (*Mercenaria mercenaria*) were readily obtained from the inland waters adjacent to Marineland, Florida.

Extraction

Circumesophageal ganglia (800 in all, about 15 g wet weight) were crudely dissected from large snails, and each was immediately put in acetone (100 ml total). The ganglia in acetone were left in the freezer until all of the tissue had been collected. The acetone was then poured off, centrifuged at 25,000 × *g* for 10 min and the supernatant saved. The ganglia were homogenized in 80% acetone (50 ml) and centrifuged (10,000 × *g* for 10 min); the pellet was extracted again, and the supernatants were combined with the original acetone. The combined acetone extracts were evaporated under reduced pressure (water aspirator) in a rotary evaporator with its bath temperature gradually being increased from room temperature. At 40°C, the water started to distill, and the remaining liquid (mainly water) was centrifuged again; the supernatant was forced through a disposable C-18 cartridge (Waters Sep-Pak). The retained material was eluted with methanol (5 ml), and this methanol was then evaporated.

Purification

The residue was taken up in 0.1 *M* acetic acid (3 ml), applied to a column of Sephadex G-15, and eluted with the same. Those fractions containing FMRFamide were detected by a radioimmunoassay (RIA) described below. The peak of immunoreactivity was centered at fraction 21 (Fig. 1A); its component fractions were combined and lyophilized. The residue was again taken up in 0.1 *M* acetic acid and run through the Sephadex column, and the immunoreactive fractions were pooled and lyophilized. A portion of this material was reserved for tryptic digestion (see below). The remainder was taken up in buffer (0.2 ml of 0.5 *M* ammonium acetate with 0.1 *M* acetate acid, pH 5.5, in n-butanol to 80% of saturation), injected onto a C-18 reserve phase column (Waters Radial-Pak), and eluted with the same solvent at 4 ml/min (Fig. 1B). Fractions 30–34 were lyophilized, redissolved in 0.5 ml of 50 m*M* ammonium acetate (pH 6.0), and applied to a small column of CM-Sephadex (0.35 × 25 cm; V_T = 2.2 ml). The columns was eluted with 10 ml of 50 m*M* ammonium acetate (pH 6.0) and then with a gradient to 500 m*M* ammonium acetate (40 ml total volume) as previously described (Price and Greenberg, 1977b) (Fig. 1C).

Characterization

An aliquot of the partially purified (Sephadex G-15) peptide, containing about half a nanomole of immunoreactivity, was digested with trypsin (10 μg) for 3 h at room temperature (pH 8.0). The material was lyophilized, then dansylated and chromatographed on polyamide TLC according to the method of Tatemoto and Mutt (1978) for amino acid alpha-amides.

An aliquot of the fully purified peptide was hydrolyzed in redistilled, constant boiling hydrochloric acid, at 108°C, for 16 h, in a sealed glass tube under nitrogen. The amino acids were determined with an automatic analyzer (Hitachi 835).

Further aliquots of purified peptide were subjected to digestion with carboxypeptidase Y (10 μg per 50 μl) at room temperature, for 20 or 90 min. Digestion was stopped by dilution with the amino acid analyzer sample-buffer (0.02 *M* HCl), and was followed by immediate injection onto the column of the analyzer.

Paper electrophoresis was done on Whatman No. 1 paper with 1% triethylamine as the buffer and a potential of 15V/cm for 70 min. The natural peptide was located by cutting the paper into sections, immersing each in RIA buffer overnight in the refrigerator, and finally taking aliquots for RIA. The peptides used as standards were synthesized by Peninsula Laboratories (San Carlos, California) and were detected on the paper strips by the Sakaguchi (Stahl, 1962) test for arginine.

Synthesis

Two peptides, pQDPFLRFamide and its 2-asparaginyl analog (pQNPFLRFamide), were synthesized on 4-methyl-benzhydrylamine resin by the solid phase method. The peptides were released from the resin and deprotected with anhydrous hydrogen fluoride. They were purified in two steps: chromatography on Sephadex G-25 with 0.05 *M* ammonium bicarbonate as elutant, followed by partition chromatography on Sephadex G-25 with n-butanol/water/acetic acid (4:1:5) as the solvent. Purity was checked by amino acid analysis and by thin-layer chromatography on silica gel [solvent: n-butanol/water/acetic acid/pyridine (15:12:3:10)], developed with both the Sakaguchi and chlorine-iodide reagents.

Radioimmunoassay (RIA)

A rabbit antiserum to a conjugate of YGGFMRFamide and thyroglobulin was used in the RIA, and iodinated YGGFMRFamide was the trace; the preparation of antiserum and trace has been described (Price, 1982). The assay was performed as follows. An aliquot (2 μl) of each fraction was transferred to a glass test tube with an automatic diluter (Micromedic model 30010) that added RIA buffer (48 μl) to give a sample volume of 50 μl. The RIA buffer contains 0.01 *M* sodium phosphate with 1% bovine serum albumin, 0.9% sodium chloride, 0.01% merthiolate, and 0.025 *M* sodium EDTA adjusted to pH 7.0; a buffer without the albumin was used for sample dilution when even traces of protein had to be prevented from contaminating the fractions. The trace (in 100 μl of buffer) was added together with diluted antiserum (also 100 μl) to each tube; the final dilution of antiserum was about 1:60,000, and there were 10,000 CPM of trace. All of the tubes were then left overnight in the cold (4°C), and 1 ml charcoal solution was added in the morning. (Charcoal solution contains 0.25% charcoal, 0.025% dextran, and 0.01% merthiolate in 0.1 *M* sodium phosphate, pH 7.5; it is stirred overnight before the first use and kept in the refrigerator thereafter.) The charcoal was centrifuged down after 15 min (2500 × *g* for 15 min), and the supernatants were decanted and counted.

Bioassays

FMRFamide-like biological activity was assayed with the radula protractor muscle of *Busycon contrarium*, and the hearts of *Mercenaria mercenaria* and *Helix aspersa*. The use of the radula protractor and clam heart bioassays were described by Price and Greenberg (1977), and that of the *Helix* heart by Kerkut and Cottrell (1963).

Chromatographic comparisons

The synthetic and natural peptides were compared in several HPLC systems and on TLC. In addition to the HPLC system used for the purification (see above), two gradient elution systems were employed with a u-Bondapak C-18 column: 0.02 *M* phosphate buffer (pH 6.5) with acetonitrile grading from 25% to 45%; and 0.2% trifluoroacetic acid with methanol from 30% to 50%. TLC was done on Eastman cellulose layers with n-butanol/acetic acid/water (4:1:2) as the solvent.

Blood determinations

One by one, snails were shelled, their aortas were cut, and the flowing blood was drawn into a calibrated pipet and quickly added to an at least 10-fold excess of acetone in a microfuge tube. Typically, an aliquot of 0.1 ml was added to 1.0 ml of acetone. The tubes were stored in the freezer for several hours and spun in a Beckman Microfuge for 3 min; the supernatants were transferred to clean tubes and dried under streams of air. Finally, the residue was dissolved in RIA buffer, and successive two-fold dilutions were assayed.

Pooled blood from 75 snails was prepared similarly, except that the blood from each snail was not measured individually, but was added to a common container of acetone. The volume of blood collected was determined by the increase in total volume. After the mixture had chilled overnight, it was centrifuged at 10,000 × *g* for 10 min; the supernatant was decanted and dried in a rotary evaporator. The residue was taken up in 0.5 ml of HPLC buffer and injected onto a Waters C-18 Bondapak column which was eluted with a gradient of acetonitrile in 0.7% trifluoroacetic acid at the rate of 2 ml/min. Fractions of 0.5 ml were collected and aliquots subjected to radioimmunoassay. Synthetic peptides were run to confirm the elution times, but only after the blood extract had been chromatographed.

RESULTS

Purification

FMRFamide-like immunoreactivity eluted from the gel-filtration (G-15) column at about three void volumes (Fig. 1A), the usual position (Price, 1986). An aliquot of this peak was subjected to trypsin digestion followed by dansylation and chromatography, and phenylalanine amide was the only amino acid alpha-amide present. Thus the material was already somewhat purified by this one step.

Several peaks of FMRFamide-like immunoreactivity eluted from HPLC (Fig. 1B). The earliest of these (at fraction 9 in Fig. 1B) co-elutes with oxidized FMRFamide, and the next one (at fraction 12 in Fig. 1B) co-elutes with FRMFamide itself. Moreover, if an acetone extract of ganglia is oxidized with either Chloramine T or hydrogen peroxide before HPLC, the peak corresponding to FMRFamide is no longer detected, the peak co-eluting with oxidized FMRFamide is augmented, and the remaining peaks are not affected (not shown). This observation supports the identification of the first two peaks and also implies that the remaining ones do not contain methionine.

The FMRFamide-like immunoreactivity eluted from CM-Sephadex just after the void volume (Fig. 1C), the same elution position as observed for the biological activity in preliminary experiments (Cottrell *et al.*, 1981).

Characterization

An amino acid analysis of the purified peptide (*i.e.*, fraction 3 from CM-Sephadex; Fig. 1C) was in substantial agreement with a corrected, earlier analysis (Price, 1982) and indicated the following composition: Arg_1, Asp_1, Glu_1, Leu_1, Phe_2, Pro_1 (Table I).

Digestion with carboxypeptidase Y led to the rapid appearance of phenylalanine, arginine, and leucine in the reaction mixture, and even by 20 min phenylalanine was

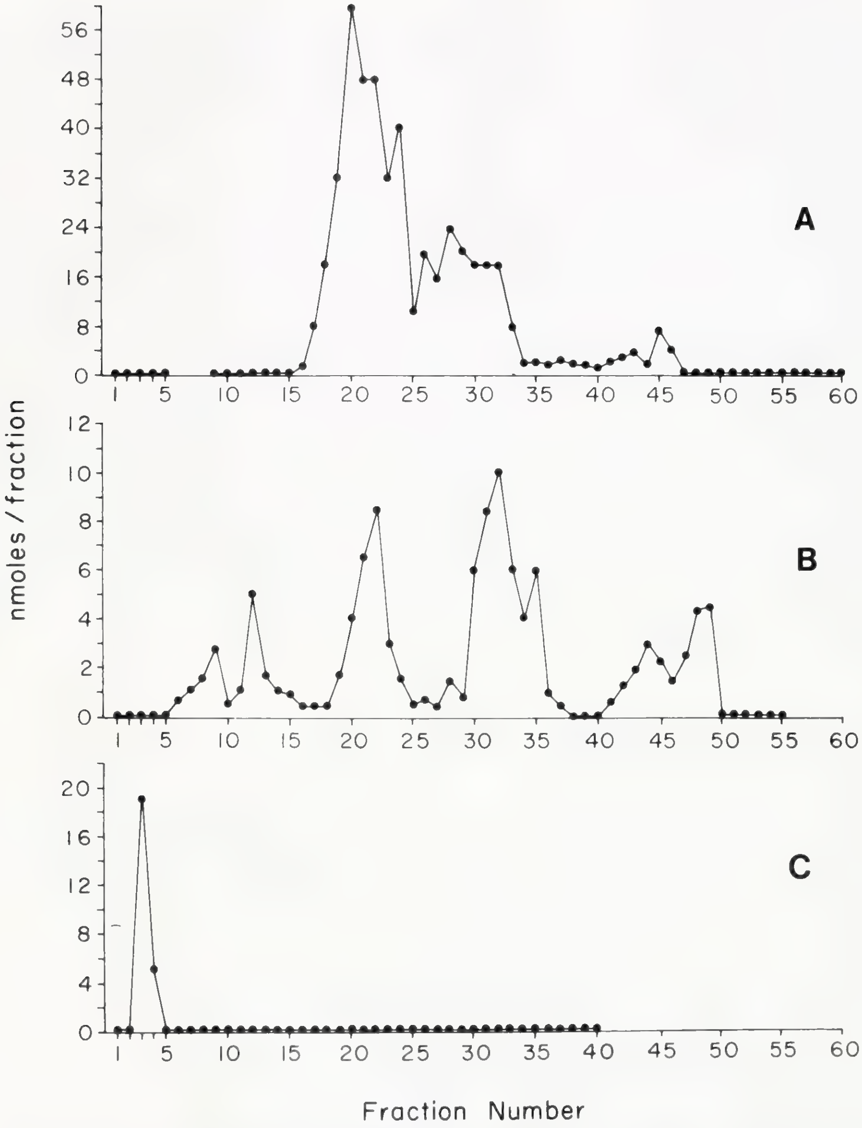

FIGURE 1. Purification of the *Helix* FMRFamide-like peptide. Crudely dissected ganglia were extracted in acetone; the supernatant was dried; the residue was dissolved in 0.1 *M* acetic acid, run through a C-18 Sep-Pak cartridge, and the adsorbed peptides were eluted with methanol (details in Materials and Methods). (A) The methanol was evaporated, and the residue was taken up in 0.1 *M* acetic acid and applied to a Sephadex G-15 column (void volume = 65 ml). Fractions of 8 ml were collected, and aliquots (2 μl) of each fraction were taken for RIA. Fractions 16–25 (the peak of activity) were pooled and lyophilized. (B) The residue was taken up in HPLC buffer and run under the conditions described in Materials and Methods. (C) Fractions 30–34 were combined, lyophilized, dissolved in a small volume (0.5 ml) of 50 m*M* ammonium acetate (pH 6.0) and applied to a CM-Sephadex column eluted with the same solvent. After collecting ten fractions of .5 ml and 5 fractions of 1.0 ml, a gradient to 500 m*M* ammonium acetate (pH 7.0) was started. Twenty fractions of 2 ml each were collected and finally pure 500 m*M* ammonium acetate was used as elutant for the last 5 fractions. The active material was completely nonretained and was recovered, almost entirely, in the third 0.5 ml fraction. In each graph, the immunoreactivity shown is the total in the fraction based on FMRFamide as standard.

TABLE I

Amino acid composition of a Helix *FMRFamide-like peptide*

Amino acid	Quantity (nmole) (1)	Quantity (nmole) (2)	Ratio to Leu (1)	Ratio to Leu (2)	Presumed # of residues
Arginine	4.40	10.3	0.99	0.99	1
Aspartic acid	4.70	11.8	1.06	1.44	1
Glycine	0.67	6.0	0.15	0.58	0
Glutamic acid	4.43	10.5	1.00	1.01	1
Isoleucine	0	1.5	0	0.14	0
Leucine	4.43	10.4	1.00	1.00	1
Phenylalanine	8.88	26.1	2.00	2.51	2
Serine	0.24	3.4	.05	0.35	0
Ammonia	6.25	n.d.	1.42	—	1 or 2
Proline	4.46	10	1.01	1	1

The purified peptides, in sealed ampules under nitrogen, were hydrolyzed in constant boiling hydrochloric acid, at 108–110°C, for 16–20 hours. Data set (1) was obtained from a peptide preparation purified as described in the section on Materials and Methods and analyzed on a sensitive amino acid analyzer (Hitachi 835-50). Data set (2) was obtained from a peptide purified by two successive isocratic reverse-phase HPLC runs from an 80% aqueous acetone extract of *Helix* ganglia and analyzed on a relatively insensitive amino acid analyzer (JEOL 6-AH). The first report of this analysis (Price, 1982) contained two errors corrected here: proline, undetected by the integrator was not reported (the value above was determined by manual integration of the record); and the values for leucine and isoleucine were inadvertently transposed. Ammonia in data set (2) could not be determined because of high background levels.

present at 1.5 times the level of leucine. After 1.5 h of digestion, free proline was present at about two-thirds the level of leucine, and phenylalanine at nearly twice the level of leucine but aspartic acid, asparagine, glutamic acid, or glutamine were not detectable. These findings, together with the previously reported ninhydrin negativity (Price, 1982) and the electrophoretic mobility reported below, allow us to deduce the sequence of this peptide, as follows.

The release of phenylalanine amide by trypsin means that the C-terminal dipeptide sequence must be -Arg-Phe-NH_2 because trypsin cleaves on the carboxyl side of basic amino acid residues, and the amide can only arise from the C-terminal amino acid. The results of carboxypeptidase Y digestion show that the last four amino acids are two phenylalanines, arginine and leucine. Moreover, leucine must occur between the phenylalanines, since the Phe/Leu ratio increases from 20 to 90 min. Thus, we must have -Phe-Leu-Arg-Phe-NH_2. Proline becomes detectable very slowly and must therefore follow the second Phe residue; hence, -Pro-Phe-Leu-Arg-Phe-NH_2. The ninhydrin-negativity means that the N-terminal must lack a free amino group, suggesting a pyroglutamyl residue and the partial sequence: pGlu-Asx-Pro-Phe-Leu-Arg-Phe-NH_2.

The identity of the Asx residue was established by paper electrophoresis. The electrophoretic conditions were chosen so that the two model peptides—pGlu-Asp-Phe-Ile-Arg-Phe-NH_2 and pGlu-Asn-Phe-Ile-Arg-Phe-NH_2—ran in opposite directions. When the *Helix* peptide and the model peptides were run in parallel, the *Helix* peptide, like the Asp^2-standard, migrated toward the anode. Thus the sequence of the *Helix* peptide must be: pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (pQDPFLRFamide expressed in the one-letter code; pyroglutamic acid derives from glutamine and is therefore coded as pQ).

Confirmation of the sequence

Both pQDPFLRFamide and its Asn^2-analog, pQNPFLRFamide, were synthesized and compared to the natural peptide chromatographically and biologically. The concentration of the natural peptide was determined by amino acid analysis. Synthetic pQDPFLRFamide and the natural peptide co-eluted in the same isocratic HPLC solvent system that had been used for the original purification; they were particularly well separated from the Asn^2-analog, as well as from FMRFamide (Fig. 2). The same two peptides also co-eluted in the two gradient elution systems tested and were again separated from the Asn^2-analog. Finally, synthetic pQDPFLRFamide had a higher Rf value (.88) than FMRFamide (.77) on TLC, substantiating an earlier value (.90) found for the ninhydrin-negative, Sakaguchi-positive peptide of *Helix* (Price, 1982).

The natural *Helix* peptide, synthetic pQDPFLRFamide, and FMRFamide are all roughly equipotent on the radula protractor muscle of the whelk, *Busycon contrarium* (Fig. 3). On the heart of *Helix aspersa,* the natural peptide and synthetic pQDPFLRFamide were again equipotent, but in this case, both were about 100 times more active than FMRFamide (Fig. 3). In contrast to their effects on the *Helix* heart, both synthetic and natural pQDPFLRFamide were about 10 times less potent than FMRFamide on the heart of the clam, *Mercenaria mercenaria* (not shown). The action of the Asn^2-analog was not distinguishable from that of pQDPFLRFamide on either the radula protractor or the *Helix* heart, but on the heart of *Mercenaria* this analog was equiactive with FMRFamide and thus distinct from pQDPFLRFamide. In summary, synthetic pQDPFLRFamide and the natural peptide were equipotent in three assay systems, so the proposed sequence is confirmed.

Comparisons of immunoreactivity

pQDPFLRFamide (natural or synthetic) is approximately equipotent to FMRFamide in our RIA (range 80–200%). Furthermore, the remaining, major, unidentified peak in *Helix,* which elutes between FMRFamide and pQDPFLRFamide on HPLC, is also roughly equipotent to FMRFamide. This means that the profiles of immunoreactivity as shown in Figure 1 reflect the actual levels of peptide in the major peaks, and that the total FMRFamide-like immunoreactivity, measured, for example, in the blood, is the unweighted sum of these three major components. The equivalent potency of these three peptides is a peculiarity of our antiserum which reacts more weakly with FLRFamide than FMRFamide, but more strongly with peptides longer than the tetrapeptide; apparently, these two effects are in approximate balance for pQDPFLRFamide. We have tested several antisera raised to conjugates to FMRFamide and find that they, too, react only weakly with FLRFamide (less than 5%); but these antisera have no preference for longer peptides and so react about equally (and weakly) with FLRFamide and pQDPFLRFamide.

Blood immunoreactivity

FMRFamide-like immunoreactivity rapidly disappears if whole *Helix* blood is allowed to stand, even on ice, yet immunoreactive FMRFamide is readily demonstrated if precautions are taken to prevent its enzymatic destruction. We used dilution into acetone to inactivate the enzymes. To see which peptides account for this immunoreactivity, we subjected an extract of about 15 ml of blood to HPLC. The pattern of peaks was the same as that seen with ganglion extracts. The peak that eluted at the expected elution time of pQDPFLRFamide contained 107 pmole, equivalent to a concentration of 7 n*M* in the original blood or about one third of the total immunoreactivity.

We measured the level of immunoreactive FMRFamide in blood from twenty-eight individual snails in various states of activity; the values ranged from 2.58 to 56.85 nanomolar with a mean of 9.5 nanomolar. We could find no significant differ-

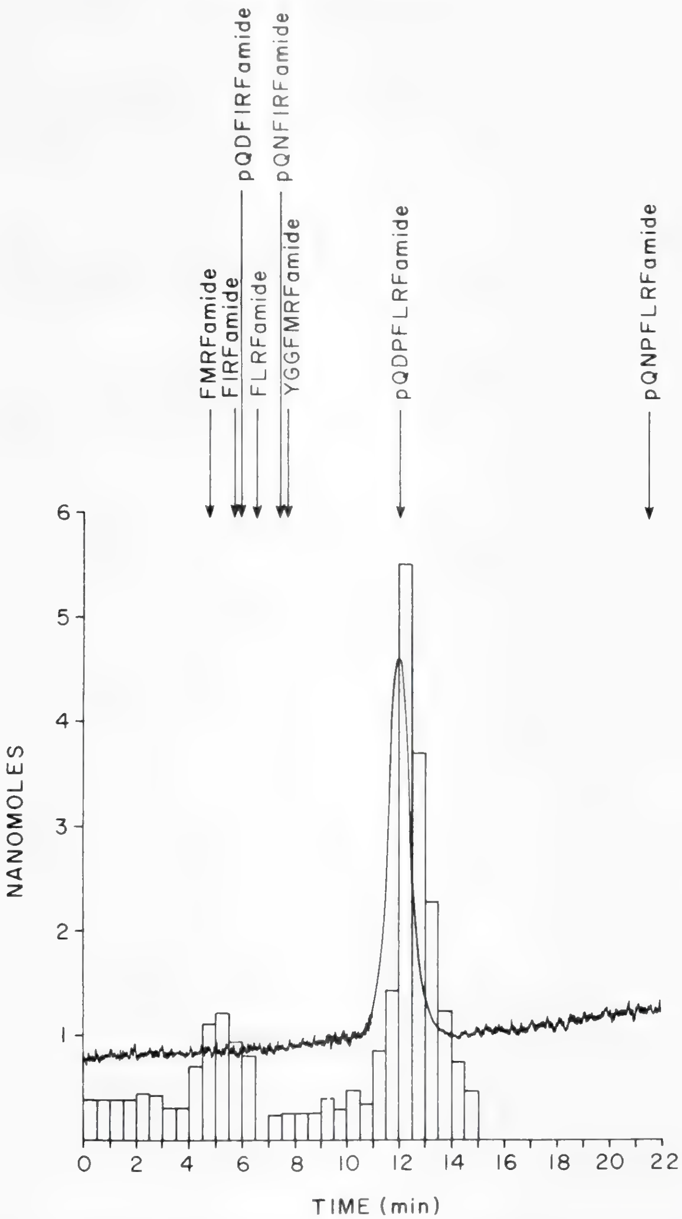

FIGURE 2. A comparison of purified and synthetic FMRFamide-like peptide from *Helix aspersa* (pQDPFLRFamide) by HPLC. Two runs were made and superimposed. In the first, the trace of ultraviolet absorbance at 257 nm was obtained for 100 nmol of synthetic peptide and is about .005 AU at the peak. In the second run, the distribution of natural peptide in the fractions was determined by radioimmunoassay (RIA) and is expressed in terms of the equivalent quantity of FMRFamide (pmol/fraction); about 180 pmol (by amino acid analysis) in 5 μl was injected onto the column (Waters Radial-Pak Micro-Bondapak C-18) which was eluted (4 ml/min) with an ammonium acetate buffer/n-butanol mixture (0.1 *M* acetic acid and 0.5 *M* ammonium acetate; butanol at 80% of saturation); 2 ml fractions were collected.

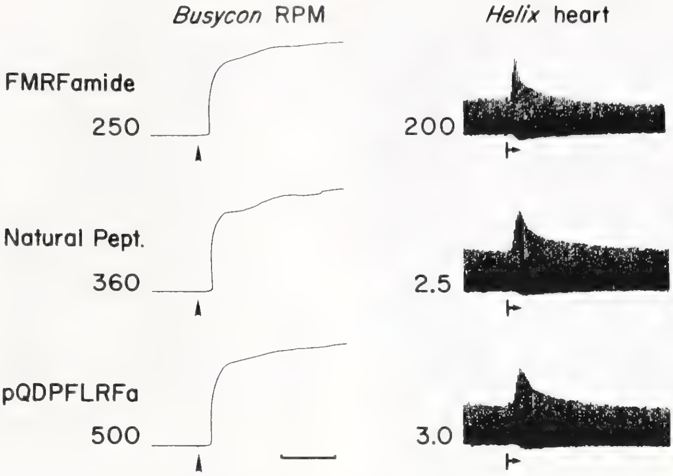

FIGURE 3. Parallel bioassay of FMRFamide and the FMRFamide-like peptide of *Helix aspersa* (pQDPFLRFamide)—both natural and synthetic—on the isolated radula protractor muscle (RPM) of *Busycon contrarium* and the ventricle of *Helix*. The records are of mechanical activity, detected with a force transducer and displayed on an inkwriting oscillograph. Doses: picomoles. The *Busycon* muscles were suspended in aerated seawater (5 ml), and the indicated doses of peptide were added. The *Helix* ventricles were suspended in, and perfused with, saline (Lloyd, 1978), and the indicated doses of peptide were dissolved in saline and injected into the perfusion stream, as follows: synthetic pQDPFLRFamide (300 μl of 10^{-8} M); natural peptide (in 200 μl); FMRFamide (200 μl of 10^{-6} M). The responses shown, for both the radula protractor and the ventricle, were chosen to be well above threshold and of equivalent height. Since the *Helix* peptide is more slowly acting than FRMFamide, the responses were matched at the point of maximum amplitude of contracture or beat. Time: 1 min.

ences between the levels in active snails and estivating snails, a possible result of our crude sampling method.

DISCUSSION

pQDPFLRFamide is only the first new analog of FMRFamide to be isolated from a mollusc and to be completely characterized; certainly others occur. For example, several peaks of immunoreactivity are evident in extracts of *Helix* ganglia (*e.g.*, Fig. 1B); and there now appear to be at least three distinct, fully processed FMRFamide-like peptides in this snail: pQDPFLRFamide, FMRFamide, and another N-terminally extended analog of FLRFamide (reviewed by Price, 1986). We conclude that *Helix* contains a family of FMRFamide-related peptides. It is now important to determine whether the members of this family occur in different neuronal populations and to compare their biological actions.

Some of the pharmacological actions of pQDPFLRFamide on tissues of *Helix* are markedly different from those of FMRFamide. For example, pQDPFLRFamide is about 100 times more potent on the heart. Also, whereas FMRFamide contracts the retractor muscles of the pharynx and tentacles, the heptapeptide relaxes them (H. K. Lehman and M. J. Greenberg, in prep.). These findings suggest that there are, in *Helix*, multiple receptor sites complementary to the various FMRFamide-like peptides. This notion had previously been proposed on the basis that analogues of FMRFamide could induce, selectively, opposing ionic currents in neurones of *Helix* (Cottrell, 1982).

The pharmacological differences between pQDPFLRFamide and FMRFamide should have a structural basis. The structure of pQDPFLRFamide differs from that of FMRFamide in two ways: it is extended at the N-terminal, and it contains a leucyl residue in place of the methionyl. Since FLRFamide and FMRFamide are equipotent in several bioassays, we suppose that the presence of leucine instead of methionine does not markedly effect the biological actions of pQDPFLRFamide. Therefore, the N-terminal extention must be critical for the recognition of this peptide by some receptor sites and hence responsible for those differences in pharmacological action that distinguish pQDPFLRFamide fom FMRFamide. This notion is supported by differences in the actions of FMRFamide and t-BOC-FMRFamide on the heart of *Helix* (Greenberg and Price, 1980), and between YGGFMRFamide and FMRFamide on *Helix* neurons (Cottrell, 1982) and on the tentacle retractor muscle of *Helix* (Cottrell *et al.*, 1983a).

Another, less specific effect of the pQDP "tail" of pQDPFLRFamide might be to protect the active FLRFamide sequence from aminopeptidase degradation. In fact, the partial sequence pQXP is found in several unrelated, biologically active peptides including TRF, gastrin, sauvigine, ranatensin, and the hydra head-activator. Thus, this N-terminal sequence may be a convergent stabilizing mechanism. An increased resistance to proteolytic degradation might explain, at least in part, the relatively high potency of pQDPFLRFamide, compared with FMRFamide, on the heart of *Helix*. A more significant consequence of its stability could be that pQDPFLRFamide acts at long range, as a hormone; likely targets in *Helix* would be the heart and the retractor muscles of the pharynx and tentacles. The tentacle muscle is known to be innervated by a FMRFamidergic neuron—C3 from the cerebral ganglion (Cottrell *et al.*, 1983b)—so in this case pQDPFLRFamide (which relaxes the muscle) may be a blood-borne antagonist of FMRFamide.

We have verified, by HPLC and RIA, that pQDPFLRFamide occurs in the blood of *Helix* and accounts for about one-third of the total FMRFamide-like immunoreactivity measured there. The mean blood concentration of pQDPFLRFamide is 3–7 nM, and the value in individual snails varies from 1 to 19 nM. These are relatively high levels of peptide, and they suggest that pQDPFLRFamide is functioning as a hormone. The targets of this hormone are presently unknown, but a likely one is the heart of *Helix* which is especially sensitive to pQDPFLRFamide. In fact, the threshold for the heptapeptide on the *in vitro* heart ranges between 1 and 10 nM, clearly overlapping the range of blood concentrations observed in individual snails. We conclude, therefore, that one hormonal role of pQDPFLRFamide is as a cardioregulator.

The individual variation in the blood levels of FMRFamide-like immunoreactivity may, in part, reflect changes in arousal state caused by our sampling procedure, but the concentration of a functional hormone should, in any event, vary widely with the physiological state of the animal. We must now inquire as to the environmental and physiological variables affecting the levels of peptide in the blood.

ACKNOWLEDGMENTS

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OSMOREGULATION, IONIC EXCHANGE, BLOOD CHEMISTRY, AND NITROGENOUS WASTE EXCRETION IN THE LAND CRAB *CARDISOMA CARNIFEX*: A FIELD AND LABORATORY STUDY

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ABSTRACT

Cardisoma carnifex in Moorea, French Polynesia, were sampled in the field and after exposure in the laboratory to either fresh- or seawater under conditions which allowed the crabs to flush their branchial chambers with the medium but not to ventilate it. Relative to field data, ionic and osmotic status of the hemolymph was virtually unchanged by exposure to freshwater, but markedly disturbed by seawater. The crabs were capable of net Na^+ and Cl^- uptake from freshwater. Water sampled from natural crab burrows was essentially freshwater. It is concluded that the population was "in equilibrium" with freshwater in the wild.

Net H^+ uptake (= base excretion) occurred in both fresh- and seawater; in freshwater there was a 1:1 relationship between net H^+ flux and strong cation minus anion flux (*i.e.*, $Na^+ + Mg^{++} + Ca^{++} + K^+ - Cl^-$). Unidirectional Na^+ and Cl^- exchanges, measured radioisotopically, were typical of euryhaline crabs in freshwater, but influxes were unusual in showing no increase in seawater. Mild dehydration caused complex alterations in these exchanges in both media, associated with small and quickly reversed changes in hemolymph composition in freshwater, but larger effects in seawater which were not reversed. High levels of ammonia in hemolymph occurred in the field but declined in the laboratory, while the level of urea was low in both situations. Both wastes were excreted into the water. Neither uric acid nor gaseous ammonia excretion were detected, and uric acid was generally not found in hemolymph. The results are discussed in relation to the ecology of this unusual animal.

INTRODUCTION

Water and electrolyte balance has now been studied extensively in the land crabs (for reviews, see Bliss, 1968, 1979; Edney, 1977; Mantel, 1979; Mantel and Farmer, 1983; Powers and Bliss, 1983). In gecarcinids, the general picture includes an ability to iono- and osmo-regulate, though far from perfectly, over a range of external salinities from 0 to >100% seawater, combined with an ability to exist for prolonged periods in the absence of free environmental water. Adaptations include reduced integumental permeability to evaporative water loss relative to aquatic crabs (*e.g.*, Herreid, 1969), tolerance of wide variations in hemolymph ionic strength and body water content (*e.g.*, Gross, 1963, 1964), and an ability to take up interstitial water from soil (*e.g.*,

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Wolcott, 1984). While the gut may have a role (Mantel, 1968), the gills are thought, though not yet proven, to be the major site of ionoregulation, as is true in aquatic crabs (Kirschner, 1979). Certainly, the branchial epithelium is rich in cells and enzymes thought to be associated with salt transport (Quinn and Lane, 1966; Copeland, 1968; Towle, 1981; Henry, 1984). The renal system (antennal gland and bladder) has been more thoroughly investigated (Gross, 1963, 1964; de Leersnyder and Hoestlandt, 1963, 1964; Gross *et al.*, 1966; Harris, 1977; Harris and Kormanik, 1981; Kormanik and Harris, 1981). It appears to be of major importance in volume regulation, minor importance in ionoregulation, and it makes a negligible contribution to osmoregulation.

Despite this intensive study, there exist only very limited data on the hydro-mineral status of gecarcinids under natural conditions in the wild (Skinner *et al.*, 1965; Gross *et al.*, 1966) and on normal rates of ionic exchange with the environment (Kormanik and Harris, 1981). The relative importance of various nitrogenous end products (ammonia, urea, uric acid) appears to be in conflict (Gifford, 1968; Horne, 1968; Henry and Cameron, 1981). The present study addresses these questions in a natural population of *Cardisoma carnifex*. We have assessed chemical conditions in the burrows, the blood chemistry of animals sampled in the field, and the changes which occurred in the latter, together with the flux rates of ions and nitrogenous wastes, when the same crabs were subsequently held in the laboratory under controlled conditions. *C. carnifex* was especially suitable for such study, since the water normally carried in its branchial chambers is intermittently changed over when an external pool is provided (Wood and Randall, 1981a). Exchange rates of substances between the whole organism and its environment could therefore be determined by periodic analysis of a closed external pool, an approach supplemented by the use of radiotracers to measure unidirectional Na^+ and Cl^- fluxes.

MATERIALS AND METHODS

Experimental animals

Land crabs [*Cardisoma carnifex;* 181.6 ± 11.4 g ($\bar{x} \pm 1$ S.E.M.); n = 39] were collected between 19:30 and 01:00 h (air temperature = 20–24°C) within 0.5 km of the shore on the island of Moorea, French Polynesia, during July and August. The crabs were actively foraging on fallen coconuts, fruits, and leaves at this time, and were generally caught after a brief chase. Within 5 min of capture, the crabs were dried and dusted with a towel as far as practical, then weighed to an accuracy of 0.2 g. No effort was made to remove water from the branchial chambers. If required, a venous hemolymph sample (0.8 ml) was immediately withdrawn from the arthrodial membrane at the base of a walking leg. The crabs were transported back to the laboratory in a closed container filled with plant material (to prevent aggression) and set up in the flux experiments within 4 h. Four groups of animals were examined. The first (n = 12) were carried through the flux protocol in a 1% seawater:99% Moorea tapwater mixture ("freshwater"; *cf.*, Table I) with hemolymph sampling at appropriate times. The second (n = 12) were treated identically in 100% seawater. The third (n = 7) were treated as the first but without hemolymph sampling, to check on possible disturbances due to this procedure. The fourth (n = 9) were simply held in freshwater for 10 days prior to a 12 h flux measurement in the standard fashion followed by a single hemolymph sample. This series, which also served as a control in a gas exchange study (Wood, Boutilier, and Randall, in prep.) thereby assessed the effects of longer term exposure to the freshwater condition in the laboratory.

Experimental protocols

In the laboratory (temperature = 24–26°C), the crabs were washed free of remaining superficial dirt by repeated brief immersion in the appropriate water (freshwater or seawater) and then placed in polyethylene buckets (22 cm diameter × 25 cm depth) fitted with lids pierced with small airholes. A water volume of $2\ l \cdot kg^{-1}$ was added, providing a depth of approximately 1 cm, which allowed the animals to draw the medium into the branchial chamber through the posterior margin of the branchiostegite, as described by Wood and Randall (1981a), but not to ventilate it. Thus the animal could only ventilate air, though some equilibration of O_2 and CO_2 between air and water phases in the branchial chambers undoubtedly occurred (Wood and Randall, 1981a; Boutilier and Wood, in prep.). As the branchial water is changed over every few minutes when an external pool is available, water samples were taken after allowing 15 min for initial flushing of the branchial chamber and again after 12 h. Both the crab and the bucket were then washed again (to remove fecal material), clean water was introduced, and the procedure was repeated so that by 48 h after capture four consecutive 12 h flux measurements had been completed. Crabs were not fed during the experiment. Fluxes determined in this manner represent movements of substances across the total body surface (*i.e.*, gills, gut, renal system, etc.). In a few instances, fresh fecal material was extracted in water and analyzed. These tests indicated that feces may have made a small contribution to measured K^+, Mg^{++}, and Cl^- losses, but were of negligible influence for other substances.

After 48 h in fresh- or seawater, the animal was then towel dried, weighed, and another hemolymph sample (0.8 ml) was taken (if required). The crab was then placed in a dry, covered bucket for an additional 48 h to evaluate the effects of mild dehydration. At the end of this period, the animal was reweighed, blood sampled (0.8 ml), and then returned to its original water for a 12 h flux determination during rehydration, followed by final weighing and blood sampling.

Unidirectional flux rates of Na^+ and Cl^- were determined during the 12 h periods immediately prior to and following dehydration by adding radiotracers (^{22}Na, ^{36}Cl; New England Nuclear; 1.0 $\mu Ci/l$) to the external water pool. Additional water samples were taken throughout these periods to monitor the disappearance of radioisotope from the water into the animal. ^{22}Na and ^{36}Cl exchanges were measured separately in half (*i.e.*, n = 5–6) of groups 1 and 2; in the third (no blood sampling) only ^{22}Na fluxes were determined. Net fluxes of acidic equivalents were also measured during these two periods in all crabs. This was feasible because defecation, which previously confounded titration alkalinity determinations by altering water buffer capacity, had ceased by these times in all animals. Changes in titration alkalinity are an important component in the calculation of acidic equivalent flux (see below).

Burrow measurements

Conditions in the burrows of an active colony in a low lying region (elevation <3 m) within 50 m of the shore were investigated during the hottest part of a sunny day (14:00–17:00 h), a period when most of the burrows were likely to be occupied. Burrows were probed with a flexible stick to which was attached a temperature sensor and a cannula for sampling water or air at known depths. Samples were analyzed for PO_2 and PCO_2 within either 10 min (air) or 60 min (water) of collection. A time series control for two sets of burrow samples showed that changes due to permeability of the syringe or endogenous metabolism by micro-organisms in the water were negligible over these periods. Water samples were centrifuged to remove silt prior to storage.

Analytical techniques

Hemolymph and water samples were frozen at −20°C for all later analyses, except for water pH and titration alkalinity measurements, which were performed shortly after collection. Hemolymph was routinely anayzed for osmolality, Na^+, Cl^-, K^+, Ca^{++}, Mg^{++}, total protein, total ammonia, urea, uric acid, and glucose. Freshwater samples were assayed for Na^+, Cl^-, K^+, Ca^{++}, Mg^{++}, ammonia, urea, and uric acid in order to determine the flux rates of these substances between the crabs and their environmental water. Seawater samples were routinely assayed only for ammonia, urea, and uric acid, because changes in electrolytes over 12 h periods proved too small to reliably measure against their very high background levels in seawater. Selected samples of both media were analyzed for titration alkalinity and osmolality. Burrow samples were assayed for PO_2 and PCO_2 using Radiometer electrode methodology as described by Wood and Randall (1981a). Osmolality was measured by vapor pressure osmometry (Wescor 5100B), Cl^- by coulometric titration (Radiometer CMT10), Na^+ and K^+ by flame photometry (Eel Mk. II or Radiometer FLM3), Mg^{++} by atomic absorption spectrophotometry (Varian 1275AA), and Ca^{++} by either atomic absorption or colorimetric reaction with 0-cresolphthalein complexone using Sigma (1981a) reagents. The former was used for all water samples and a few hemolymph samples, the latter for most hemolymph samples; the two techniques were cross-validated. Micro-modifications of commercial diagnostic kits were used for the spectrophotometric assay of glucose (0-toluidine method: Sigma, 1980), urea (diacetyl monoxine method; Sigma, 1981b), uric acid (uricase/phosphotungstate method; Sigma, 1981c), hemolymph total protein (Lowry method; Sigma, 1982b), and hemolymph total ammonia, (1-glutamic dehydrogenase/NAD method; Sigma 1982a). Total ammonia in water was determined by a micro-modification of the salicylate-hypochlorite method of Verdouw *et al.* (1978). Different ammonia assays were used for water and hemolymph as the simpler salicylate-hypochlorite method gave spurious values for hemolymph; the two assays were cross-validated on water standards. Titratable alkalinity was determined by titration of air-equilibrated water samples (10 ml) to pH = 4.00 with 0.02 *N* HCl using a Gilmont micrometer burette as described by McDonald and Wood (1981). ^{22}Na and ^{36}Cl radioactivities were determined by counting 5-ml water samples or 0.2-ml hemolymph samples (in 4.8 ml water) in 10 ml ACS fluor (Amersham) on a Beckman LS 250 scintillation counter operated with a maximum window. At the 33% water content, the ACS fluor gives a very high and uniform counting efficiency (~94%) for both of these highly energetic radioisotopes. Quenching is a constant, as determined by addition/recovery tests, and therefore cpm (after correction for background), rather than dpm, could be used directly in radiotracer calculations (see below).

Calculations

Net flux rates (J_{net}) of each substance (*e.g.*, X) were calculated as:

$$J_{net} = \frac{([X]_i - [X]_f) \times V}{t \times W} \tag{1}$$

where i and f refer to initial and final concentrations in $\mu equiv \cdot ml^{-1}$ or $\mu mol \cdot ml^{-1}$, V the volume of the external water pool in ml, t the elapsed time in h, and W the body weight in kg. Thus net losses by the animal have a negative sign, and net gains a positive sign. By reversing the i and f terms, the net titratable acidity flux was calculated from the titratable alkalinities.

The net flux of acidic equivalents (J_{net}^{H+}) was calculated as the sum of the titratable acidity and ammonia fluxes, signs considered, which derives from the original principles outlined by Maetz (1973). In brief, an increase in titratable acidity of the water results from either an addition of acidic equivalents and/or a removal of basic equivalents by the animal. A decrease in titratable acidity results from the opposite processes. Ammonia is produced metabolically as NH_3. If ammonia is excreted as NH_3, it will trap protons in the medium (as NH_4^+) which will escape titration in the alkalinity measurement. Alternately, if ammonia is excreted as NH_4^+, then the protons carried out on NH_4^+ represent an addition of acidic equivalents to the water which will again escape titration. Thus the sum of the titratable acidity and ammonia fluxes yields the net acidic equivalent flux. It should be pointed out that this procedure does not distinguish between NH_3 and NH_4^+ fluxes, nor between a net excretion of acidic equivalents and a net uptake of basic equivalents, or *vice versa*. Fortunately neither matters in terms of the net acid-base status of the animal.

Unidirectional influxes (J_{in}) of Na^+ and Cl^- were calculated from the disappearance of radioactivity from the external water using a logarithmic model:

$$J_{in} = \frac{(\ln Q^*_{out_i} - \ln Q^*_{out_f}) \cdot Q_{out}}{t \cdot W} \qquad (2)$$

where Q^*_{out} (in cpm) was the total amount of radioactivity in the external pool at each time and Q_{out} (in μequiv) was the average amount of the ion in the external pool, with appropriate correction for backflux when internal specific activity exceeded 5% of external activity (*cf.*, Kirschner, 1970). Because of the very large size of Q_{out} in seawater, changes in Q^*_{out} over time were very small, and thus measurements of J_{in} were less accurate than in freshwater. Therefore, a second, independent estimate of J_{in} in seawater was obtained from the appearance of radioactivity in the hemolymph over 12 h, using an equation analogous to (2) and assuming that the distribution volumes [radiospaces, see equation (4) below] for ^{22}Na and ^{36}Cl in seawater animals were the same as in freshwater animals. In all treatments, values of J_{in} determined by the two techniques were not significantly different, and well correlated in individual determinations ($r = 0.92$, $P < 0.01$, $n = 16$); mean values are presented in Results.

Unidirectional outflux rates (J_{out}) could then be calculated by the conservation equation (in freshwater only):

$$J_{net} = J_{in} - J_{out} \qquad (3)$$

Internal distribution volumes (V_{int}) for ^{22}Na and ^{36}Cl were calculated from the equation of Mayer and Nibelle (1969):

$$V_{int} = \frac{Q^*_{out_i} - Q^*_{out_f}}{\Delta C \times W} \qquad (4)$$

where ΔC represented the increase in radioactivity of hemolymph ($cpm \cdot ml^{-1}$) between the start and end of the flux period. The total exchangeable internal pools (*i.e.*, Na_{int}^+, Cl_{int}^-) could then be estimated. For example:

$$Na_{int}^+ = V_{int}^{Na^+} \times [Na^+]_h \qquad (5)$$

where $[Na^+]_h$ was the concentration of sodium in hemolymph in $\mu equiv \cdot ml^{-1}$.

Data have been expressed as means ± 1 S.E.M. (n) unless otherwise stated. The significance ($P < 0.05$) of differences between means was assessed with Student's two tailed *t*-test, using either a paired (within group comparisons) or unpaired design (between group comparisons) as appropriate; data were transformed (logarithmic function) when necessary to match variance ratios.

RESULTS

Field observations

Crab colonies were common in low lying swampy or grassy areas, such as disused coconut plantations, within ~0.5 km of the shore. The animals exhibited mainly crepuscular or nocturnal activity, retreating to their burrows during the heat of the day, though daylight foraging increased greatly after rainfall. The burrows were simple, largely vertical holes, with one or occasionally two changes in angle (Fig. 1) and a diameter just large enough to allow the crab to enter sideways. The elaborate "castles" described for this species in the Andaman Islands (Silas and Sankarankutty, 1960) were not seen. A typical burrow was about 1 m in depth, and the bottom 15–25% was invariably filled with muddy water (Fig. 1). The air at the base of the burrows was significantly reduced in PO_2 and elevated in PCO_2 relative to the surface, presumably reflecting the presence of the crab and lack of mixing in these long, narrow blind-ended tubes. The water was markedly hypoxic and hypercapnic, even relative to burrow air (Fig. 1), and contained substantial levels of ammonia, and low levels of both urea and uric acid (Table I), suggesting that the water was used by the crab to flush the branchial chambers, as a ventilatory medium, and/or as a depository for urine. With one exception (burrow #7), burrow water was essentially freshwater (Table I, *cf.*, Table II), despite the fact that all samples were taken within 50 m of the shore. Even in burrow #7, which was less than 10 m from shore, groundwater was less than 15% seawater. Ionic ratios in individual samples were highly variable, and did not reflect those in seawater (*cf.*, Table II). Again this may have reflected the influence of the crab. For example, Mg^{++}, which was relatively high in burrow waters, is reported to be the one cation which is concentrated above hemolymph levels in *Cardisoma* urine (de Leersnyder and Hoestlandt, 1964).

Table II compares the composition of hemolymph sampled from freshly captured crabs in the wild to that of the freshwater and seawater used in flux experiments in the laboratory. Note that with the exception of Mg^{++} and nitrogenous wastes, the freshwater used was similar to burrow water (*cf.*, Table I). In the field, *Cardisoma* hemolymph was osmotically equivalent to about 70% seawater, with major, approximately equal, contributions from Na^+ and Cl^-. The total measured concentration of

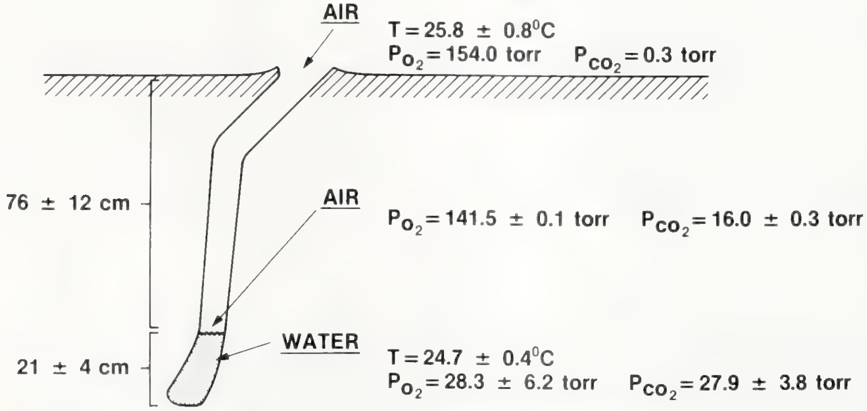

AIR
T = 25.8 ± 0.8°C
P_{O_2} = 154.0 torr
P_{CO_2} = 0.3 torr
76 ± 12 cm
AIR
P_{O_2} = 141.5 ± 0.1 torr
P_{CO_2} = 16.0 ± 0.3 torr
21 ± 4 cm
WATER
T = 24.7 ± 0.4°C
P_{O_2} = 28.3 ± 6.2 torr
P_{CO_2} = 27.9 ± 3.8 torr

FIGURE 1. Dimensions and conditions in the burrows of *Cardisoma carnifex*. Means ± 1 S.E.M. n = 7–9 different burrows for all measurements except air P_{O_2} and P_{CO_2}, where n = 2.

TABLE I

The composition of water sampled from the burrows of Cardisoma carnifex

Burrow	Osmolality (mOsm · kg^{-1})	Na^+ (mequiv · l^{-1})	Cl^- (mequiv · l^{-1})	K^+ (mequiv · l^{-1})	Ca^{++} (mequiv · l^{-1})	Mg^{++} (mequiv · l^{-1})	Ammonia (μmol · l^{-1})	Urea (μmol · l^{-1})	Uric acid (μmol · l^{-1})	pH
1	18	2.35	3.40	0.85	1.48	10.02	172	10.5	8.6	7.07
2	21	3.33	10.40	0.80	1.79	3.31	253	12.3	6.1	6.94
3	26	1.94	2.90	0.74	1.01	8.81	141	12.9	4.0	6.99
4	41	5.98	7.00	0.32	5.39	2.48	38	13.1	5.3	7.10
5	17	4.03	4.90	0.39	1.16	6.17	290	21.5	0.7	7.01
6	16	1.77	2.58	0.17	1.44	6.31	57	14.8	1.1	6.91
7	(–)	(*38.58*)	(*72.00*)	(*1.36*)	(*8.95*)	(*35.92*)	(*193*)	(*44.9*)	(*12.9*)	(*7.02*)
$\bar{x}$[1]	23	3.23	5.20	0.55	2.05	6.18	159	14.2	4.3	7.00
S.E.M.[1]	4	0.65	1.24	0.11	0.68	1.21	41	1.6	1.2	0.03

[1] Excluding the data of burrow 7.

TABLE II

A comparison of the composition of hemolymph of Cardisoma carnifex *sampled in the field with that of freshwater and seawater used in flux experiments in the laboratory*

	Hemolymph	Freshwater[1]	Seawater[1]
Osmolality (mOsm · kg^{-1})	733 ± 6 (24)	18 ± 1 (10)	1055 ± 3 (11)
Na^+ (mequiv · l^{-1})	351.3 ± 2.1 (24)	6.59 ± 0.04 (93)	496.7 ± 7.2 (38)
Cl^- (mequiv · l^{-1})	359.4 ± 3.6 (24)	6.36 ± 0.12 (91)	567.6 ± 1.9 (38)
K^+ (mequiv · l^{-1})	8.3 ± 0.1 (24)	0.20 ± 0.01 (91)	10.6 ± 0.3 (38)
Ca^{++} (mequiv · l^{-1})	22.0 ± 0.6 (24)	0.70 ± 0.04 (92)	20.9 ± 0.3 (37)
Mg^{++} (mequiv · l^{-1})	18.8 ± 0.8 (24)	1.24 ± 0.04 (92)	106.6 ± 1.2 (37)
Ammonia (μmol · l^{-1}	2673 ± 424 (24)	41 ± 5 (92)	59 ± 6 (68)
Urea (μmol · l^{-1})	226.8 ± 30.0 (24)	5.9 ± 0.4 (92)	5.6 ± 0.5 (68)
Uric acid (μmol · l^{-1})	<3.0 (24)	<0.5 (93)	<0.5 (38)
Glucose (μmol · l^{-1})	160 ± 38 (24)	–	–
Protein (g · 100 ml^{-1})	4.98 ± 0.54 (24)	–	–
Titration alkalinity (μequiv · l^{-1})	–	971 ± 31 (42)	2527 ± 31 (24)
pH	–	7.03 ± 0.12 (42)	8.01 ± 0.17 (24)

[1] Initial measurements at start of flux periods.
Means ± 1 S.E.M. (n).

osmolytes in the hemolymph was 742 mmol · l^{-1}, in comparison to a measured osmolality of 733 mOsm · kg^{-1} (Table I). As one major osmolyte ($SO_4^{=}$) was unmeasured, while ion pairing and protein binding undoubtedly decreases osmotic activity in hemolymph, agreement between the two values seems quite reasonable. Hemolymph Mg^{++} was maintained at less than 20% seawater levels, while the Ca^{++} concentration was equal to that in full strength seawater. All major electrolytes were 15 to 60× freshwater levels. Ammonia was by far the most abundant of the measured nitrogenous wastes in blood, reaching very high concentrations (9.42 mmol · l^{-1} in one crab). Urea was less than one tenth the ammonia level, but was detected in all animals. The detection limit for uric acid in hemolymph was ~3 μmol · l^{-1}; in only 2 of 24 crabs were levels above this value (5, 6 μmol · l^{-1}) observed.

Laboratory observations

Blood was taken from two groups in the field prior to the flux protocol in the laboratory in fresh- and seawater respectively, a third was put through the flux protocol in freshwater without blood sampling, and the fourth was examined after 10 days in freshwater in the laboratory without prior sampling. There were no significant differences in any measured parameters in the hemolymph samples taken in the field from the freshwater and seawater flux groups (Figs. 2, 3, 4, 5).

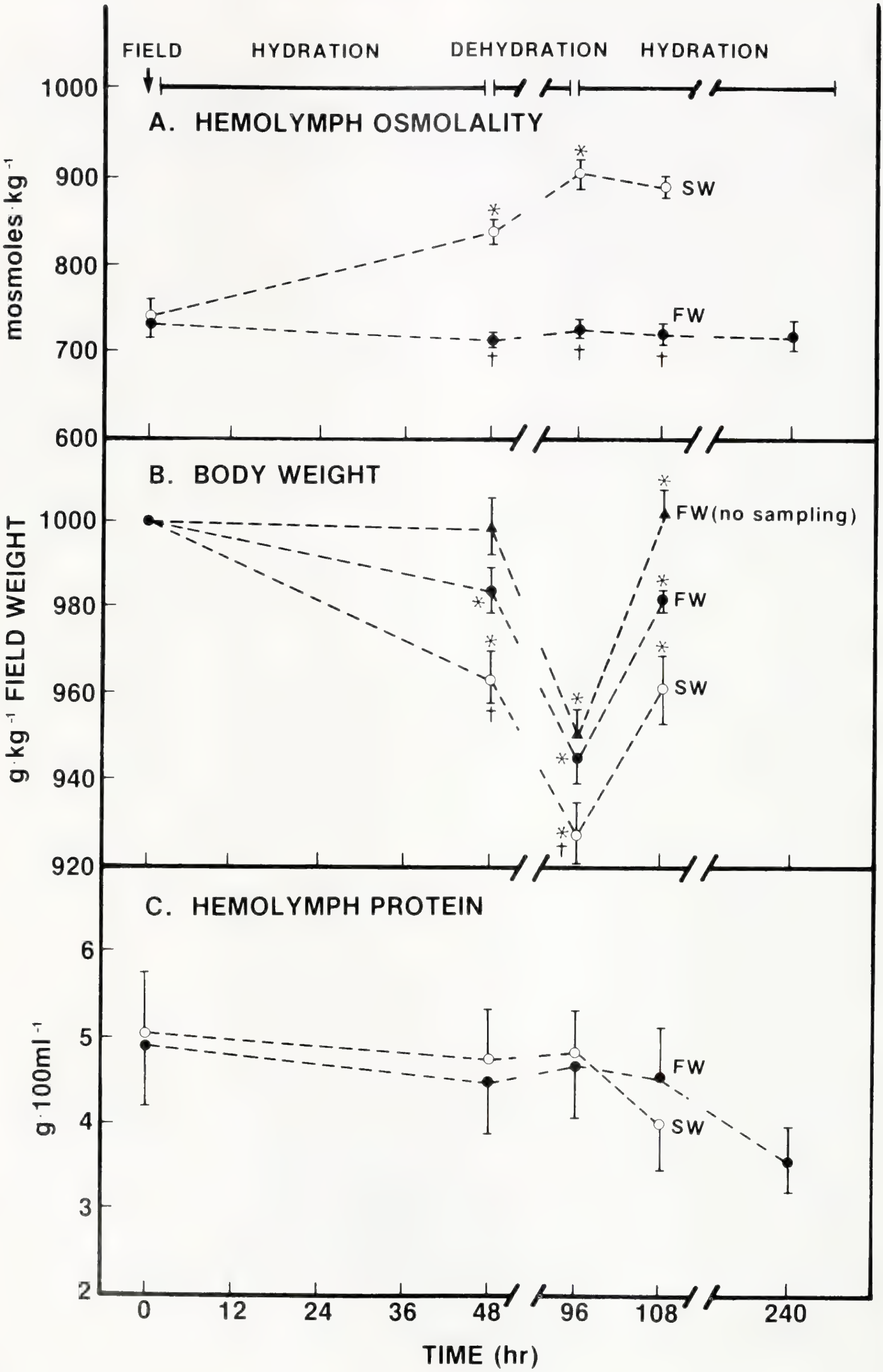
Ionic and osmotic status of hemolymph. Exposure to freshwater for 48 h after capture caused minimal net gain or loss of water, as witnessed by the constancy of hemolymph osmolality, total protein (mainly hemocyanin), and body weight, the latter in non-sampled animals only (Fig. 2). Inasmuch as the crabs defecated, some weight loss was to be expected, so a small net uptake of water may have occurred. (The crabs from which blood samples were taken lost about 15 $g \cdot kg^{-1}$ over the same period, only 40% of which could be directly attributed to the weight of the removed sample, so there may have been some stress resulting in loss of more weight.) Similarly, the crabs maintained unchanged levels of Na^+, Cl^-, Ca^{++}, and Mg^{++} in hemolymph (Fig. 3A–D). The only electrolyte to change significantly after 48 h in freshwater was K^+, which decreased by 28% (Fig. 3E). After 10 days in freshwater, hemolymph osmolality (Fig. 2A), protein (Fig. 2B), and Cl^- (Fig. 3B) still remained at levels seen in the field, while Na^+ had decreased by only 5% (Fig. 3A). However, Ca^{++} had fallen by ~25% (Fig. 3C) and both K^+ and Mg^{++} by ~50% (Fig. 3D, E) by this time.

Exposure to seawater after capture produced a very different pattern. After 48 h, body weight had declined by over twice as much (~36 $g \cdot kg^{-1}$), suggesting a net loss of water (Fig. 2B). Hemolymph osmolality (Fig. 2A), Na^+, Cl^-, and Ca^{++} (Fig. 3A–C) increased by 14–20%, and Mg^{++} by ~40% (Fig. 3D). However K^+ was well regulated (Fig. 3E), although it should be noted that levels of K^+ in seawater were only slightly higher than those in hemolymph (Table II).

Dehydration for 48 h decreased body weight by ~40 $ml \cdot kg^{-1}$ in both freshwater groups, an effect which was fully reversed during 12 h of rehydration (Fig. 2B). This may have reflected changes in branchial chamber water, true body water, or both. Greatly increased flushing of the branchial chambers occurred during the rehydration period. While hemolymph osmolality, protein (Fig. 2A, C), Mg^{++}, and K^+ (Fig. 3D, E) were unaffected, very small but significant increases (2–3%) and subsequent decreases in Na^+ and Cl^- (Fig. 3A, B) were seen. Interestingly, Ca^{++} rose by about 8%, but only during the rehydration period (Fig. 3C).

The weight changes during dehydration and rehydration in seawater were similar (Fig. 2B), but disturbances in hemolymph composition more pronounced than in freshwater. Osmolality (Fig. 2A), Na^+, and Cl^- levels (Fig. 3A, B) rose by 6–9% and did not fall again during rehydration. Other osmolytes were not significantly affected.

Ionic exchange rates and radiotracer studies. During the first 12 h after capture, crabs maintained significant net uptakes (~ +400 $\mu equiv \cdot kg^{-1} \cdot h^{-1}$) of both Na^+ and Cl^- (Fig. 4A, B) from freshwater. These rates subsequently declined, becoming significantly negative at 36–48 h. Throughout this same period, the crabs exhibited net losses of Ca^{++}, Mg^{++}, and K^+ (Fig. 4C–E), though these fluxes were much smaller and showed no significant trends over time. After 48 h dehydration, $J_{net}^{Na^+}$ and $J_{net}^{Cl^-}$ returned to significantly positive values similar to those immediately after capture (Fig. 4A, B), while Mg^{++} and K^+ losses were significantly reduced (Fig. 4D, E); Ca^{++} balance was unaffected (Fig. 4C). These responses were generally similar regardless of whether blood samples had been taken, suggesting that they were not due to ionic depletion


FIELD
HYDRATION
DEHYDRATION
HYDRATION
A. HEMOLYMPH OSMOLALITY
mosmoles·kg⁻¹
1000
900
800
700
600
SW
FW
B. BODY WEIGHT
g·kg⁻¹ FIELD WEIGHT
1000
980
960
940
920
FW(no sampling)
FW
SW
C. HEMOLYMPH PROTEIN
g·100ml⁻¹
6
5
4
3
2
FW
SW
0
12
24
36
48
96
108
240
TIME (hr)

as a result of hemolymph withdrawal. After 10 days in freshwater, the crabs were in positive balance for Na^+ and Cl^- (Fig. 4A, B), while Ca^{++}, Mg^{++}, and K^+ fluxes, although still negative, were significantly lower than during the first 48 h.

Net flux rates for these ions could not be directly measured in seawater. However the hemolymph data suggest net uptakes of all ions except K^+ over the experimental period (Fig. 3).

Determinations of $J^{H^+}_{net}$, calculated as the sum of the titratable acidity and ammonia fluxes (Fig. 8A), signs considered, are summarized in Table III. After 36–48 h "hydration" in the laboratory, all groups exhibited a net acidic equivalent uptake (= base excretion). After 10 days in freshwater, $J^{H^+}_{net}$ was significantly reduced but still positive. Dehydration for 48 h had no effect in freshwater, but reduced $J^{H^+}_{net}$ to approximately zero in seawater. Figure 5 illustrates that over a wide range of flux rates, there was an approximate 1:1 relationship between $J^{H^+}_{net}$ and the measured strong cation minus anion fluxes (*i.e.*, $Na^+ + K^+ + Ca^{++} + Mg^{++} - Cl^-$) in *Cardisoma.*

Unidirectional flux determinations with ^{22}Na and ^{36}Cl yielded almost identical values for sampled and non-sampled groups, so the data have been combined in Figure 6. During the 36–48 h post-capture period ("hydration" in Fig. 6), $J^{Cl^-}_{in}$ ($\sim +1500$ $\mu equiv \cdot kg^{-1} \cdot h^{-1}$) was approximately twice as large as $J^{Na^+}_{in}$ in freshwater. For both ions, J_{out} exceeded J_{in}, resulting in negative net balance. After 48 h dehydration, this balance became positive during rehydration, but whereas $J^{Na^+}_{out}$ was reduced without significant change of $J^{Na^+}_{in}$, $J^{Cl^-}_{in}$ was stimulated without significant alteration of $J^{Cl^-}_{out}$.

Interestingly, despite the very different gradients in the two situations, unidirectional Na^+ and Cl^- influx rates in seawater were similar to those in freshwater during "hydration." However in contrast to the freshwater picture, both $J^{Na^+}_{in}$ and $J^{Cl^-}_{in}$ approximately tripled after 48 h dehydration in seawater (Fig. 6).

Internal distribution volumes (V_{int}) and total exchangeable pools were similar for Na^+ and Cl^- in freshwater ($V_{int} \cong 35\%$ body weight; pool $\cong 125$ $mequiv \cdot kg^{-1}$) and were unchanged after the dehydration/rehydration treatment (Table IV). Volumes and pools could also be estimated at the immediate end of the 48 h dehydration period, assuming no radioisotope had been lost from the animal. These were only ~10% higher than initial values (data not shown), suggesting that these treatments caused no marked alterations in internal distributions. Estimates in seawater (data not shown) were imprecise due to specific activity limitations (see Materials and Methods) but did not differ significantly from the freshwater data.

Metabolites. After 48 h in the laboratory in either fresh- or seawater, the glucose concentration of hemolymph increased approximately 5-fold from the very low levels measured in the field (Fig. 7C). Thereafter, glucose declined, irrespective of the dehydration/rehydration treatment; by 10 days in freshwater, the low levels seen in the field were restored.

Conversely, the ammonia concentration of hemolymph declined from the very high levels seen in the field (Fig. 7A). Ammonia excretion rates decreased over a similar time course (Fig. 8A). Sampled and non-sampled groups behaved similarly, so the data have been combined in Figure 8 for clarity. Hemolymph concentrations

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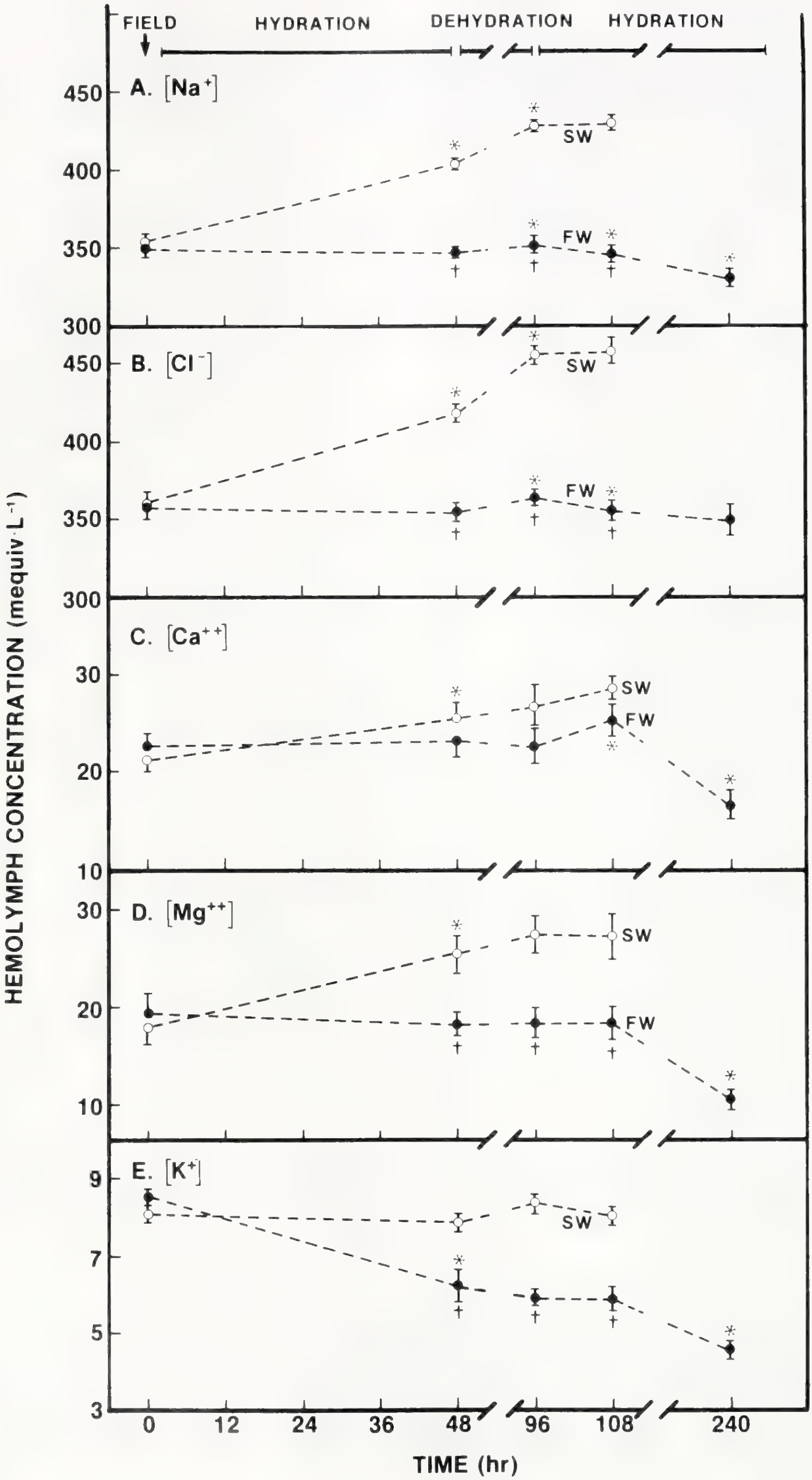
FIGURE 2. Changes relative to field measurements in (A) hemolymph osmolality, (B) body weight, and (C) hemolymph protein concentration in *Cardisoma carnifex* exposed to either freshwater or seawater in the laboratory. See text for details of treatment regimes. Means ± 1 S.E.M. n = 6–12 at each point. t = significant difference ($P \leq 0.05$) *between* the freshwater and seawater groups at a comparable time. * = significant difference ($P \leq 0.05$) *within* each group; each mean is tested relative to the preceding one, except for the 10-day (240 h) freshwater hydration value, which is compared with the 48-h freshwater hydration value.


FIELD
HYDRATION
DEHYDRATION
HYDRATION
A. [Na⁺]
B. [Cl⁻]
C. [Ca⁺⁺]
D. [Mg⁺⁺]
E. [K⁺]
SW
FW
450
400
350
300
30
20
10
9
7
5
3
0
12
24
36
48
96
108
240
HEMOLYMPH CONCENTRATION (mequiv·L⁻¹)
TIME (hr)

and flux rates after 10 days in freshwater were similar to those seen after 48 h. A comparable pattern of decline occurred in seawater, though net ammonia fluxes were only ~35% of those in freshwater. During the 12 h rehydration period immediately following 48 h dehydration, ammonia excretion rates increased to the levels seen immediately after capture in both groups (Fig. 8A), though this was not accompanied by significantly elevated hemolymph levels (Fig. 7A).

Levels of urea in hemolymph were much lower than those of ammonia, and unlike the latter, remained unchanged under all treatment conditions (Fig. 7B). Excretion rates of urea were generally less than 20% of the simultaneously measured ammonia fluxes and unaffected by experimental conditions or salinity (Fig. 8B). Uric acid could not be detected (detection limit $\cong 3\ \mu\text{mol} \cdot \text{l}^{-1}$) in the hemolymph under any laboratory condition, and there was no measurable uric acid excretion to either freshwater or seawater (detection limit in water $\cong 0.5\ \mu\text{mol} \cdot \text{l}^{-1}$).

To check the possibility that the animals might excrete ammonia gas by direct volatilization to the atmosphere, crabs were enclosed in jars (in the absence of water) through which air was pumped at $\sim 1\ \text{l} \cdot \text{min}^{-1}$. The air passed first through a 0.1 *N* HCl trap (for humidification and removal of any ambient ammonia), then to the crab jar, then to a second identical ammonia trap. In one 6-h and one 48-h experiment, no ammonia was detected in the outflow trap, despite considerable ambient ammonia accumulation in the inflow trap.

DISCUSSION

The purpose of sampling crabs first shortly after capture in the wild and then subsequently after exposure to defined conditions in the laboratory was to assess their "natural" condition of water and electrolyte balance. The present population of *Cardisoma carnifex* in Moorea appeared to be essentially "in equilibrium" with freshwater when sampled on land in the field. Subsequent exposure to freshwater in the laboratory caused little change in most osmotic and ionic parameters, in contrast to seawater exposure. These physiological data are supported by the presence of freshwater in the burrows, where the animals spend at least half their time. It is likely that the crabs carry this burrow water with them in the branchial chambers when foraging above ground (*cf.*, Wood and Randall, 1981a). It is not clear whether this pattern can be extrapolated to the genus as a whole, for comparable information in the literature is fragmentary. Gross *et al.* (1966) sampled *C. carnifex* "in less than 12 h of capture" from a mangrove area in Madagascar and found blood osmolalities much higher than the present data and closer to those in animals "equilibrated" with seawater than with freshwater. Burrow water was not collected. Kormanik and Harris (1981) reported hemolymph osmolality identical to the present data in "freshly collected" *C. carnifex* from the Palau Archipelago; burrow waters ranged from 35 to 85% seawater, though it is not clear that these were *C. carnifex* burrows. Herreid and Gifford (1963) found that inland burrows of *C. guanhumi* in Florida contained freshwater but reported no blood data from crabs in the field.

The differences in hemolymph ionic and osmotic composition associated with fresh- or seawater exposure are in basic agreement with previous observations on *Cardisoma* sp. (Herreid and Gifford, 1963; de Leersnyder and Hoestlandt, 1963, 1964;

FIGURE 3. Changes relative to field measurements in (A) sodium, (B) chloride, (C) calcium, (D) magnesium, and (E) potassium concentrations of hemolymph in *Cardisoma carnifex* exposed to either freshwater or seawater in the laboratory. Other details as in legend of Figure 2.

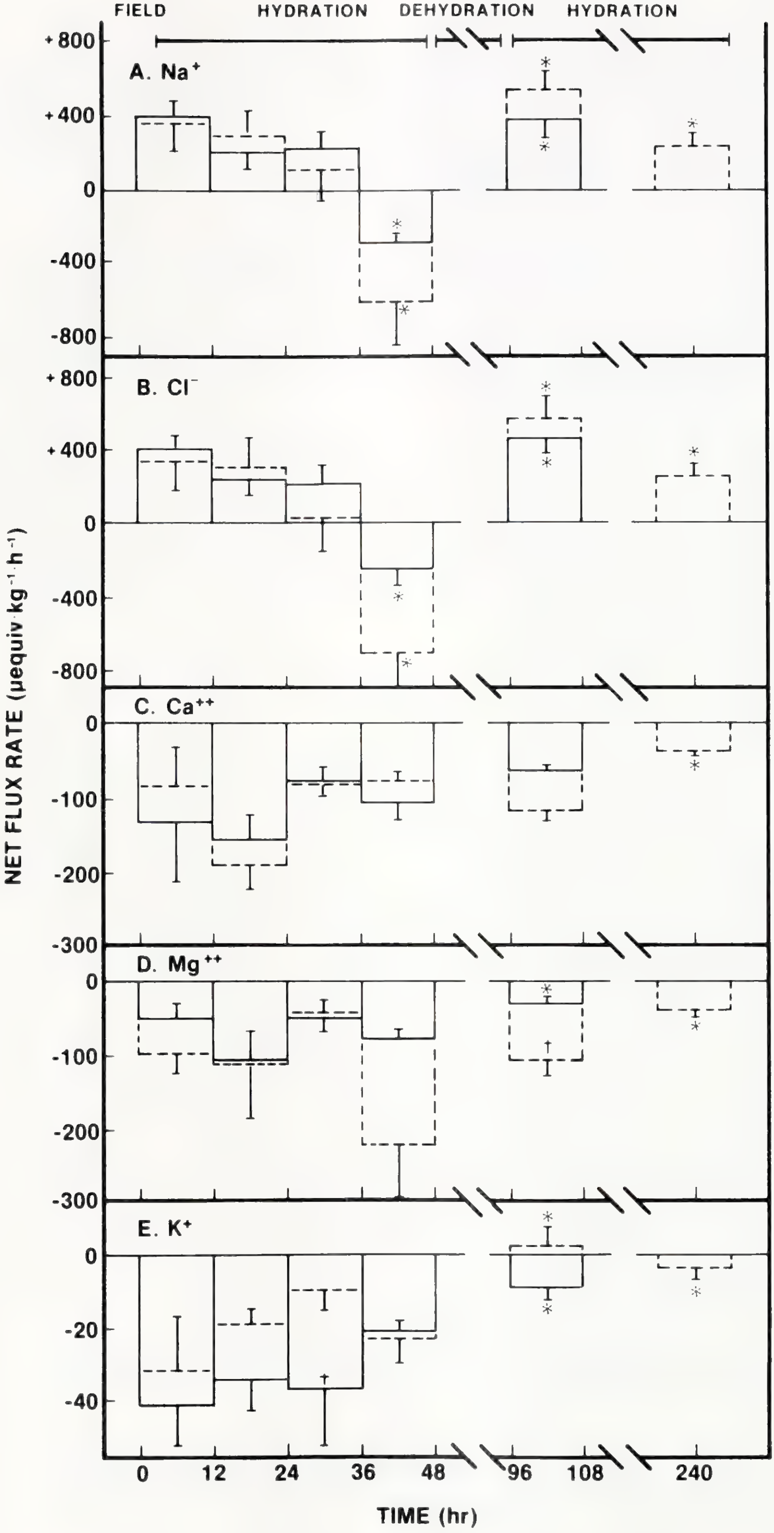

FIELD
HYDRATION
DEHYDRATION
HYDRATION
A. Na⁺
B. Cl⁻
C. Ca⁺⁺
D. Mg⁺⁺
E. K⁺
+800
+400
0
-400
-800
-100
-200
-300
-20
-40
NET FLUX RATE (μequiv·kg⁻¹·h⁻¹)
0
12
24
36
48
96
108
240
TIME (hr)

TABLE III

Net acidic equivalent fluxes ($J^{H^+}_{net}$: $\mu equiv \cdot kg^{-1} \cdot h^{-1}$) *in* Cardisoma carnifex *held in either freshwater or seawater under various conditions in the laboratory*

	Hydration (36–48 h)	After 48 h dehydration (96–108 h)	Hydration (240 h)
Freshwater (no blood sampling)	+278.6 ± 73.4 (7)	+201.4 ± 37.3 (7)	+116.3 ± 23.0* (9)
Freshwater (blood sampling)	+190.1 ± 26.1 (10)	+248.2 ± 31.8 (10)	–
Seawater (blood sampling)	+120.1 ± 30.1 (12)	+16.1 ± 32.3*,† (12)	–

* Significantly different ($P \leq 0.05$) from 36–48 h hydration value.
† Significantly different ($P \leq 0.05$) from comparable freshwater values.
Means ± 1 S.E.M. (n).

Gross *et al.*, 1966; Kormanik and Harris, 1981). To our knowledge, however, the present data are the first to demonstrate a net uptake of Na$^+$ and Cl$^-$ from freshwater in land crabs. Renal NaCl loss rates in *Cardisoma* sp. in freshwater have been variously estimated as -400 $\mu equiv \cdot kg^{-1} \cdot h^{-1}$ (Kormanik and Harris, 1981) to -6500 $\mu equiv \cdot kg^{-1} \cdot h^{-1}$ (de Leersnyder and Hoestlandt, 1964). The former appears more reasonable, for our unidirectional estimates with radiotracers indicate whole body J_{out} values of only -1500 $\mu equiv \cdot kg^{-1} \cdot h^{-1}$, even in a period of negative J_{net} (Fig. 6), and some of this undoubtedly occurred at extra-renal sites. Thus positive J_{net} values of $+100$ to $+400$ $\mu equiv \cdot kg^{-1} \cdot h^{-1}$ (Fig. 4) must reflect J_{in} values at least several-fold greater at the transport sites, a conclusion confirmed by the radiotracer J_{in} measurements ($+700$ to $+1500$ $\mu equiv \cdot kg^{-1} \cdot h^{-1}$, Fig. 6). Although transepithelial potentials were not determined, active transport is almost certainly involved in view of the large chemical gradients between freshwater and hemolymph (Table II). The gills, and possibly the gut, are the likely sites of this active transport (see Introduction).

In contrast to the constancy of Na$^+$ and Cl$^-$ in hemolymph during freshwater exposure, there were decreases in K$^+$ (after 48 h), Mg^{++} and Ca^{++} (after 10 days) (Fig. 3), and continuous net losses of these ions to the medium (Fig. 4). As the herbivorous diet of *Cardisoma* is rich in these electrolytes, the higher hemolymph levels in the field are probably maintained by dietary intake rather than uptake from water (*cf.*, Gross and Holland, 1960). However the latter source cannot be entirely discounted, for Ca^{++} and Mg^{++} levels were somewhat higher in burrow water than in the freshwater used in the laboratory experiments (Tables I, II).

While seawater exposure caused marked physiological disturbances, these were

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FIGURE. 4. Whole body net flux rates over 12 h periods of (A) sodium, (B) chloride, (C) calcium, (D) magnesium, and (E) potassium in *Cardisoma carnifex* exposed to freshwater in the laboratory. See text for details of treatment regimes. Positive values represent net uptakes, negative values net losses. Means ± 1 S.E.M. Data from hemolymph-sampled groups (n = 11–12; solid lines) and non-sampled groups (n = 6–7; dotted lines) are shown separately. t = significant difference ($P \leq 0.05$) *between* the sampled and non-sampled groups at a comparable time. * = significant difference ($P \leq 0.05$) *within* each group for the following comparisons: the 36–48-h hydration value is tested against the 0–12-h hydration value; the 96–108-h rehydration value is tested against the 36–48-h hydration value; and the 10-day (240 h) hydration value is tested against the 36–48-h hydration value.

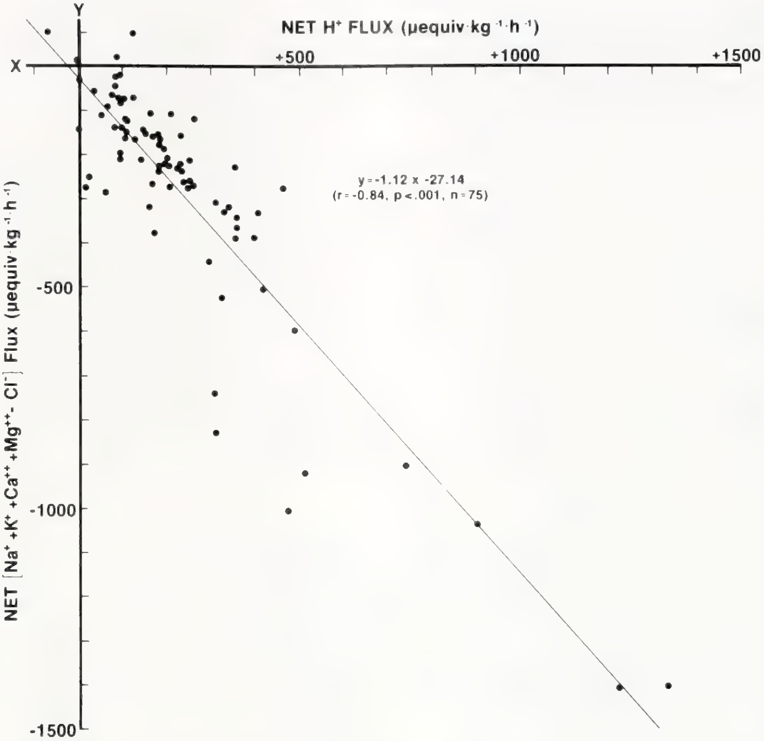

FIGURE 5. The regression relationship between the whole body net flux rate of acidic equivalents ($J_{net}^{H^+}$; X) and the simultaneously measured difference (Y) between the whole body net flux rates of strong cations ($Na^+ + K^+ + Ca^{++} + Mg^{++}$) and strong anions ($Cl^-$) in *Cardisoma carnifex* exposed to freshwater in the laboratory. Positive values represent net uptakes; negative values net losses. Data from hemolymph-sampled and non-sampled groups during both hydration and rehydration have been combined. The slope of the regression relationship is not significantly different from 1.0, and the intercept is not significantly different from origin ($P > 0.05$).

well within the tolerance of the animal. Indeed we observed that *C. carnifex* would readily enter seawater in the wild, and one group survived in the laboratory in 100% seawater for three weeks without mortality. Previous reports concur (Gifford, 1962; de Leersnyder and Hoestlandt, 1963, 1964; Gross *et al.*, 1966), and emphasize the role of the renal system in selective excretion of Mg^{++} and $SO_4^=$. Nevertheless, hemolymph levels of these ions do rise considerably in seawater (*e.g.*, Fig. 2, also de Leersnyder and Hoestlandt, 1964), and their neuro-muscular depressant action may explain the reduced activity commonly observed.

Forty-eight hours dehydration had minimal influence on the ionic composition of hemolymph in freshwater equilibrated crabs (Figs. 2, 3), despite a weight loss (~40 $g \cdot kg^{-1}$) equivalent to about 20% of the extra-cellular fluid volume (~195 $ml \cdot kg^{-1}$; Kormanik and Harris, 1981). This suggests that much of the loss was from branchial water, and that the crab used this pool to regulate the osmolality of hemolymph during dehydration, thereby emphasizing the value of this pool. While the weight loss was the same during dehydration in seawater equilibrated crabs, there were substantial further increases in hemolymph osmolality and ions, which were not corrected during rehydration (Figs. 2, 3). This illustrates the disadvantage of seawater for *Cardisoma.* Other studies of dehydration in *C. carnifex* (Harris and Kormanik, 1981; Burggren and McMahon, 1981) employed much more severe, nearly terminal stress conditions (3–4 fold greater weight loss) and so are not directly comparable.

Unidirectional flux rates for Na^+ and Cl^- for *C. carnifex* in freshwater (Fig. 6) were typical of euryhaline aquatic crabs, but the finding of unchanged $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ in seawater was most unusual. Exchange rates one to two orders of magnitude higher normally occur, and are thought to at least partly reflect large exchange diffusion components (Kirschner, 1979). While most workers have measured efflux rates as an index of exchange in seawater because of the technical difficulty of influx determinations, we adopted the opposite approach for consistency with the freshwater measurements. We are confident of our results, as two independent methods (radiotracer disappearance from the water, appearance in the animal) yielded the same values ($J_{in}^{Na^+} \cong +1000$; $J_{in}^{Cl^-} \cong +1300\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). From the hemolymph ion data (Fig. 3) and the space estimates (Table IV), net Na^+ and Cl^- uptake rates of $\sim +400\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ can be estimated over the first 48 h in seawater, indicating values of $J_{out}^{Na^+} \cong -600$ and $J_{out}^{Cl^-} \cong -1000\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. Kormanik and Harris (1981) directly measured $J_{out}^{Na^+}$ at $-1600\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ in *C. carnifex* "immersed in seawater," so the two studies are in reasonable agreement. Thus Na^+ and Cl^- exchange rates in *Cardisoma* are atypically low in seawater, perhaps reflecting an absence of exchange diffusion and/or simply reduced flushing of the branchial chambers. The tripling of exchange seen after dehydration (Fig. 6) could result from the greatly increased flushing noticeable during rehydration.

At rest, *C. carnifex* has an unusually low respiratory quotient ($\cong$0.58), suggesting the fixation of some respiratory CO_2, perhaps as $CaCO_3$ for carapace growth as earlier hypothesized (Wood and Randall, 1981a, b; Wood, Boutilier, and Randall, unpub.). Such a strategy would necessitate the excretion of two H^+ ions for every CO_2 stored as $CO_3^{=}$. This situation has recently been documented in the immediately post-moult aquatic blue crab, *Callinectes sapidus,* during the period of rapid carapace mineralization (Cameron and Wood, 1985). In intermoult *C. carnifex* at normal resting metabolic rates ($\dot{M}_{CO_2} \cong 1400\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; $\dot{M}_{O_2} \cong 2400\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Wood and Randall, 1981a), the difference between R.Q = 0.58 and the theoretical minimum R.Q $\cong$ 0.70 (lipid metabolism) would imply the fixation of at least $280\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ of respiratory CO_2, yielding a $J_{net}^{H^+}$ of at least $-560\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. In contrast, the present measurements show a net H^+ uptake ($J_{net}^{H^+} = +100$ to $+300\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Table III), suggesting either that the theory is wrong or that the predicted net H^+ excretion in *C. carnifex* is swamped by metabolic base production. As most herbivorous diets are strongly base producing (Hills, 1973), this is quite possible; clearly further work is needed to settle the matter.

The only other measurements of acidic equivalent fluxes in crabs using comparable methodology are also in *Callinectes sapidus,* which is both carnivorous and euryhaline. Interestingly, this species in intermoult condition exhibits $J_{net}^{H^+} \cong +380\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (Cameron, 1979), declining to $\cong 0\ \mu\text{equiv} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ in seawater (Wood and Cameron, 1985), a comparable trend to that seen in *C. carnifex* (Table III).

According to the concept of strong ion difference (Stewart, 1978), solutions separated by membranes (*e.g.*, the body fluids of the crab and the external water) can only interact in acid-base terms by processes which alter the values of their dependent variables, which are the difference in concentration between strong cations and strong anions, the P_{CO_2}, and the total weak acid present (mainly protein). If the latter two are constant, as was likely the case in the present flux experiments, a change in the

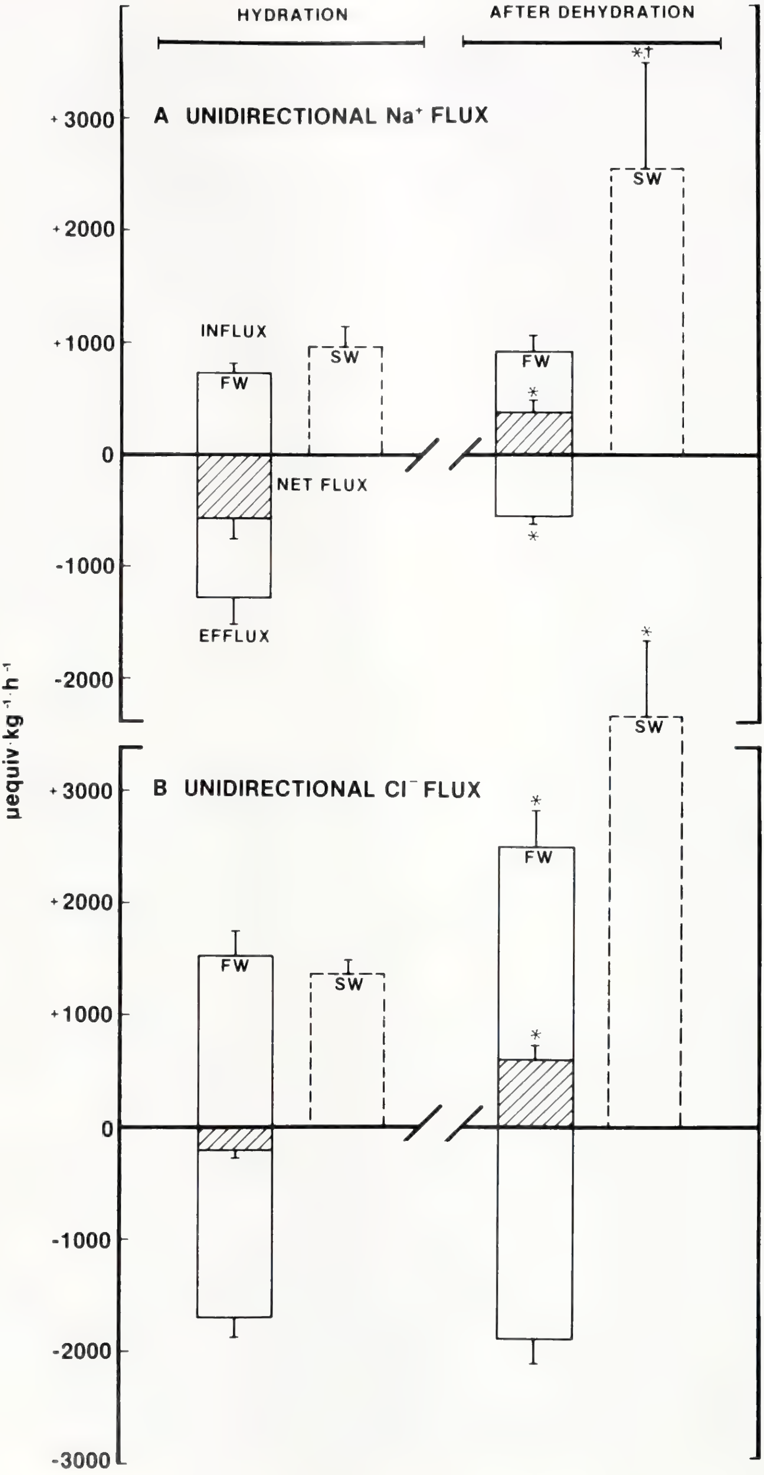
HYDRATION
AFTER DEHYDRATION
A UNIDIRECTIONAL Na+ FLUX
B UNIDIRECTIONAL Cl⁻ FLUX
INFLUX
EFFLUX
NET FLUX
FW
SW
*
*,†
+3000
+2000
+1000
0
-1000
-2000
-3000
μequiv·kg⁻¹·h⁻¹

TABLE IV

Internal distribution volumes (V_{int}) and total exchangeable internal pools (Na^+_{int}, Cl^-_{int}) as estimated with ^{22}Na and ^{36}Cl in Cardisoma carnifex *held in freshwater*

	Hydration (*i.e.*, 48 h)	After 12 h rehydration (*i.e.*, 108 h)
$V^{Na^+}_{int}$ (ml · kg^{-1})	363.8 ± 12.7 (5)	359.0 ± 6.8 (3)
Na^+_{int} (mequiv · kg^{-1})	123.64 ± 4.15 (5)	120.64 ± 2.28 (3)
$V^{Cl^-}_{int}$ (ml · kg^{-1})	337.7 ± 21.6 (5)	352.6 ± 24.4 (4)
Cl^-_{int} (mequiv · kg^{-1})	121.01 ± 8.59 (5)	127.82 ± 9.77 (4)

There were no significant differences between Na^+ and Cl^- values at comparable times, or between comparable hydration and rehydration values.

Means ± 1 S.E.M. (n).

strong ion difference generated by a transmembrane flux will constrain an equal and opposite flux of acidic equivalents. The observed 1:1 relationship between $J^{H^+}_{net}$ and the measured strong cation minus anion fluxes in *C. carnifex* in freshwater (Fig. 5) is in agreement with this strong ion difference concept (Stewart, 1978), and suggests that the fluxes of all quantitatively important strong electrolytes have been accounted for. (The only electrolyte of likely importance whose flux was not measured was $SO_4^=$.) A similar relationship has been documented recently in the freshwater fish gill, taking only Na^+ and Cl^- into account (Wood *et al.*, 1984). In *Cardisoma*, however, the relationship was rather weak unless other ions were included (K^+, Ca^{++}, Mg^{++}), emphasizing the quantitatively greater importance of these exchanges in crabs.

In direct contrast to the current finding of high ammonia, low urea, and virtually no uric acid in hemolymph in *C. carnifex* in Moorea (Table II, Fig. 7), Henry and Cameron (1981) found high levels of uric acid, low levels of ammonia, and undetectable urea in the same species in the Palau Archipelago. We employed the same assays for urea and uric acid as Henry and Cameron (1981), and indeed confirmed that they accurately measured these substances in human body fluids, even when diluted with crab hemolymph, so this is not the source of the difference. Our own measurements of low ammonia levels in the Palau crabs (Wood and Randall, 1981b) were also in agreement with Henry and Cameron (1981), so the crabs appear fundamentally different in the two habitats. Interestingly, the present data are very similar to those of Horne (1968) on *C. guanhumi* (comparable high ammonia, low urea, and only very

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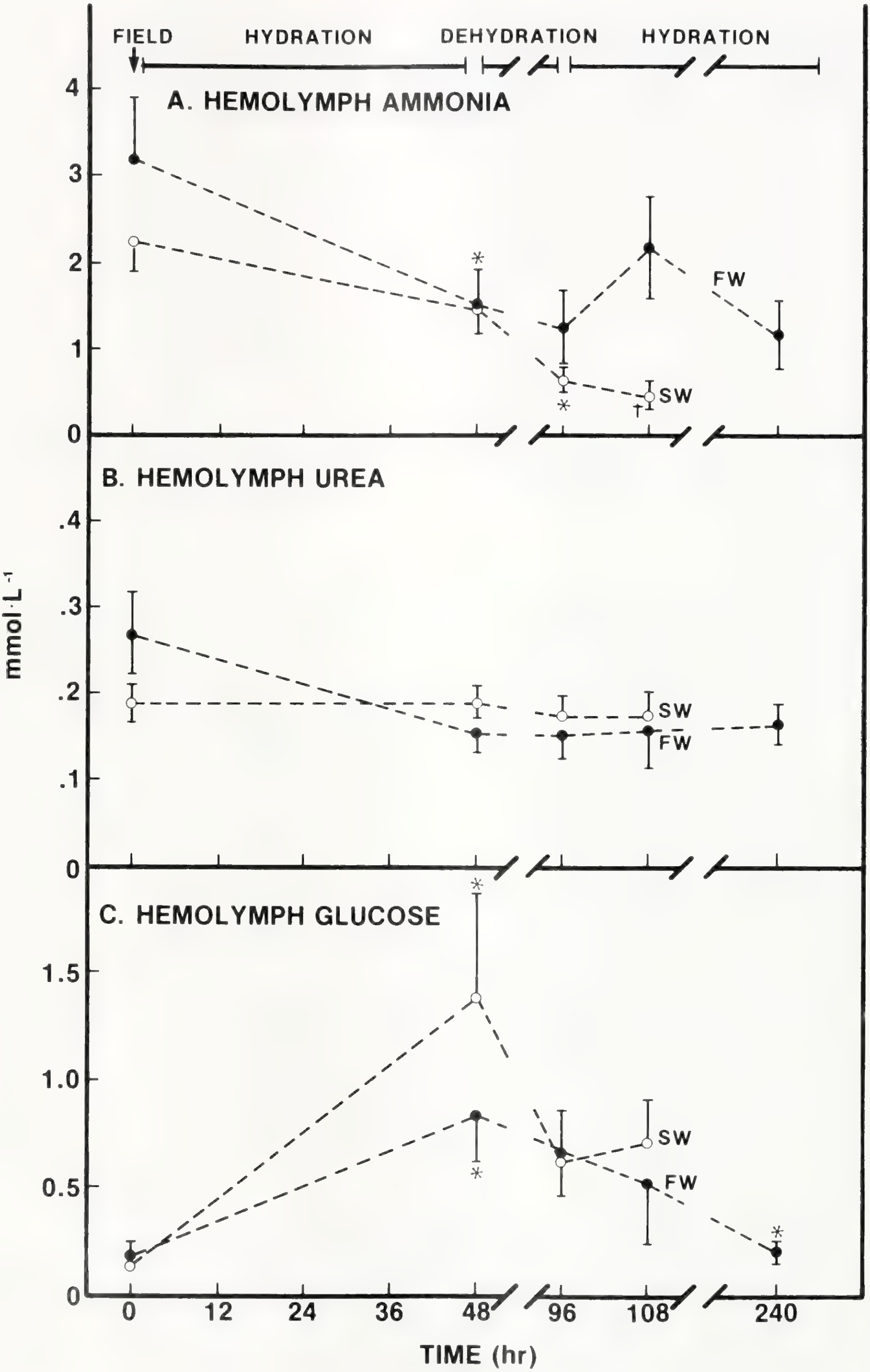
FIGURE 6. Unidirectional measurements with radiotracers of (A) whole body sodium exchanges and (B) whole body chloride exchanges in *Cardisoma carnifex* exposed to either freshwater (influxes, effluxes, net fluxes, solid lines) or seawater (influxes only, dotted lines) in the laboratory. Means ± 1 S.E.M. Data did not differ significantly between hemolymph-sampled and non-sampled groups in freshwater and have been combined. n = 11 for sodium exchange in freshwater; n = 5 for chloride exchange in freshwater; n = 6 for sodium exchange in seawater; n = 6 for chloride exchange in seawater. "Hydration" measurements were taken at 36–48 h; "after dehydration" measurements at 96–108 h. t = significant difference ($P \leq 0.05$) *between* freshwater and seawater groups under the same condition; * = significant difference ($P < 0.05$) *within* each group under different conditions.


FIELD
HYDRATION
DEHYDRATION
HYDRATION
A. HEMOLYMPH AMMONIA
B. HEMOLYMPH UREA
C. HEMOLYMPH GLUCOSE
mmol·L⁻¹
4
3
2
1
0
.4
.3
.2
.1
0
1.5
1.0
0.5
0
FW
SW
*
†
0
12
24
36
48
96
108
240
TIME (hr)

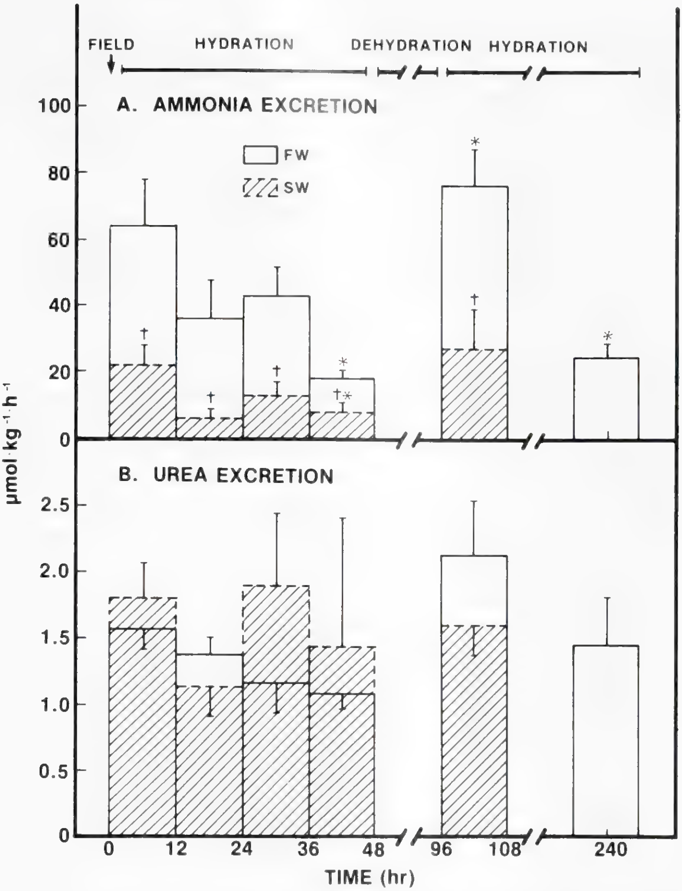


FIGURE 8. Whole body net excretion rates over 12 h periods of (A) ammonia and (B) urea in *Cardisoma carnifex* exposed to either freshwater (open bars, solid lines) or seawater (cross-hatched bars, dotted lines) in the laboratory. See text for details of treatment regimes. Means ± 1 S.E.M. Data did not differ significantly between hemolymph-sampled and non-sampled groups in freshwater and have been combined. n = 16–17 for freshwater; n = 11–12 for seawater. t = significant difference ($P \leq 0.05$) *between* freshwater and seawater groups at a comparable time. * = significant difference ($P \leq 0.05$) *within* each group for the following comparisons: the 36–48-h hydration value is tested against the 0–12-h hydration value; the 96–108-h rehydration value is tested against the 36–48-h hydration value; and the 10-day (240 h) hydration value is tested against the 36–48-h hydration value.

low levels of uric acid) but not those of Gifford (1968) on *C. guanhumi* (high ammonia and high uric acid, urea not assayed). Gifford also noted copious white deposits of uric acid in the hemocoel. Similar white deposits were observed (without analysis) in the Palau specimens of *C. carnifex* (Henry and Cameron, 1981; Wood and Randall, 1981a) but did not occur in the Moorea animals. An anecdotal comment by Bright (1968) may offer some resolution of this dilemma. Bright noted that in *C. crassum*

FIGURE 7. Changes relative to field measurements in (A) hemolymph ammonia concentration, (B) hemolymph urea concentration, and (C) hemolymph glucose concentration in *Cardisoma carnifex* exposed to either freshwater or seawater in the laboratory. Other details as in legend to Figure 2.

the amount of uric acid varied with the habitat, and that none was present when the supply of groundwater was substantial, as was the case in the current study. Possibly nitrogen metabolism in *Cardisoma* sp. is very flexible, and biochemical variation can be brought on by seasonal changes or environmental pressures. Uric acid was found in burrow water in Moorea (Table I); this could reflect leaching of uric acid from cast carapaces from an earlier dry season, for crabs moult in the burrows (Bliss, 1968), or could simply originate from sources other than the crabs.

The very high blood levels of ammonia (1–10 $\text{mmol} \cdot \text{l}^{-1}$) seen in *C. carnifex* in the field (Table I, Fig. 7A) and also in *C. guanhumi* (Gifford, 1968; Horne, 1968) are within the range considered toxic for most vertebrates (*e.g.*, Warren, 1958; Hillaby and Randall, 1979). These levels fell and ammonia excretion occurred when the crabs were held in the presence of water, with subsequent increases after a period of dehydration (*e.g.*, Fig. 8A). However from the work of Horne (1968) and our own more extensive studies on dehydration (Wood, Boutilier, and Randall, in prep.), it is clear that feeding history and metabolic rate are other important influences, and that excretion rates and hemolymph levels are not always closely related. Thus high ammonia levels and excretion rates (and low glucose levels) of field animals (Table II, Figs. 7, 8) probably reflected high metabolic rates (*i.e.*, activity) associated with feeding. Changes in these parameters during holding in the laboratory may in turn have resulted from a combination of starvation and hydration effects. The much lower excretion rates of animals held in seawater (Fig. 8) may be indicative of a depression of metabolic rate. Alternately it could be associated with an enlargement of the free amino acid pool for tissue fluid balance (*cf.*, Henry and Cameron, 1981).

The rates of total nitrogenous waste excretion recorded in *C. carnifex* in the present study (10–100 $\mu\text{mol N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) were similar to those reported for *C. guanhumi* (Horne, 1968; Gifford, 1968), but only 5–30% of those in a vertebrate of comparable size (*e.g.*, Wright and Wood, 1985). While there is general agreement that nitrogen metabolism is reduced in land crabs, several authors have suggested that the animals might excrete undetected ammonia gas by direct volatilization to the atmosphere (Campbell *et al.*, 1972; Henry and Cameron, 1981). The tests for this in the present investigation, while limited, indicated that *C. carnifex* does not excrete ammonia by volatilization. In summary, the present and previous studies demonstrate that nitrogen metabolism in *Cardisoma* is unusual, complex, and labile. There is a clear need for a comprehensive investigation of the topic employing a thorough search for alternate end products and excretion routes, and careful control of feeding and hydration states.

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GROWTH OF *OCTOLASMIS COR* (AURIVILLIUS, 1892) ON THE GILLS OF *SCYLLA SERRATA* (FORSKAL, 1755)

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ABSTRACT

A unique experimental procedure to determine the growth of *Octolasmis cor* used to advantage the suspended crab cage operation in the Straits of Johore, Singapore. This allowed growth measurements of a previously inaccessible symbiotic organism in a natural situation. The resulting data suggest that growth to sexual maturity is rapid, occurring within a two-week period. It is speculated that this is an adaptation to an ephemeral substrate which allows *O. cor* to maximize its reproductive capacity by increasing egg production and the number of potential hosts.

INTRODUCTION

Although there are well-documented reports on the growth of certain species of Balanomorpha (Barnes and Powell, 1953; Costlow and Bookout, 1953; Mawatari *et al.*, 1954; Barnes, 1956; and Daniel, 1958), there are relatively few such reports on the Lepadomorpha (Evans, 1958; Skerman, 1958; Page, 1983), and none on epizoic barnacles such as *Octolasmis* which live attached to certain Crustacea. *Scylla serrata* (Forskal, 1755) was the host selected for this study because in earlier work (Jeffries *et al.*, 1982), all of the 45 individuals examined had *Octolasmis* in their gill chambers.

S. serrata is indigenous to the Johore Straits (Chuang, 1961), an estuary fed by several rivers and rich in plankton flora and fauna (Khoo, 1967; Tham *et al.*, 1970). Large numbers are also imported to Singapore by air from nearby Southeast Asian countries and held in cages suspended in the Johore Straits until ready for market. The purposes of this research was to measure the growth rate of *Octolasmis cor* from cyprid to large adult on the gills of *S. serrata* under nearly natural conditions and to consider the implications for coevolution of the epizoite and its host.

MATERIALS AND METHODS

Methods of managing the hosts are essential prerequisites for the study of growth in epizoic organisms. Clean substrates must be provided for attachment of epizoites, the time of attachment must be known or at least inferable, and the hosts must be maintained under natural or nearly natural conditions. The crab cage operation in the Straits of Johore provided a unique opportunity to meet these conditions. In turn, this allowed the development of techniques to determine the rate of growth of a species of *Octolasmis* which, because they occur within the gill chambers of decapods, are inaccessible to direct observation and sequential measurement.

From late May until the middle of August 1983, *S. serrata* were obtained within 48 hours of arrival in Singapore by air freight from Indonesia. Crabs 83 mm to 115

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mm in carapace width and within five days of ecdysis ("pre-molts") were selected for the experiment. They were recognized by a marked color contrast between the white ventral thorax (sternum) and the dark blue abdomen; and by a distinctive condition most noticeable on the underside of the postero-lateral carapace (pterygostomian), in which the old exoskeleton is thin and brittle and yields to pressure, and the epimeral suture begins to separate.

Each pre-molt crab was assigned a number and placed alone in a wire cage (30 cm × 17 cm × 14 cm) with mesh size of 14 mm by 20 mm. The cage was suspended mid-way between the surface and the bottom in the Johore Straits about 300 m north of the terminus of Lim Chu Kang Road. The overall depth at the experiment site was about 10 m at mean high water. On an annual basis the salinity ranges from 28.00 to 30.10‰, and sea surface temperatures range from 28.4 to 30.8°C (Khoo, 1966). Each crab was checked every 24 hours until it molted. By designating the molt time to be mid-way between the observed molt and the previous inspection, it was possible to insure that the designated time was within 12 hours of the actual molt. The exuviae was removed from the cage, the crab was maintained in its cage at mid-water for 24 hours, then lowered to the bottom for the remainder of the experiment. Crabs were maintained at the bottom for various periods of time as substrates for growing *Octolasmis cor. O. cor* cyprids occur in the plankton in the Johore Straits and are commonly found on hosts exposed for more than 24 hours. The shortest interval from molting to the time the crab was sacrificed was 36 hours. At 24-hour increments additional crabs were sacrificed up to a maximum of 372 hours (15.5 days).

RESULTS

Of 70 *S. serrata* processed during the experiment, 66 were observed to host *Octolasmis.* The crabs were sacrificed, their gills removed and preserved in 10% neutral formalin. Later 837 metamorphosed *O. cor* were located, counted, and measured as described previously (Jeffries and Voris, 1983).

The intervals over which the crabs were exposed to infestation by *O. cor* are arranged into periods of 48 hours in Table I and the largest *O. cor* observed for each period is given. For purposes of this study individual barnacles of large size, not averages, were used to calculate growth rates since they could be assumed to be among the earliest to attach to the recently molted crabs. Calculation of their growth rates, made over the full exposure time, thus yields the most conservative estimates.

The largest barnacle (4.719 mm capitular length) among the 837 observed, was

TABLE I

The number and maximum capitular length of O. cor *obtained from the gills of* S. serrata *which had been exposed 36–360 hours post-ecdysis*

Hours	Number of crabs	Number of cyprids	Number metamorphosed	Largest capitular length (mm)
0–48	5	106	2	1.00
60–108	14	314	122	1.86
120–168	13	103	221	2.57
180–228	12	30	220	4.00
240–288	10	12	132	3.72
300–348	8	10	113	4.72
360–	4	19	27	3.58
	66	594	837	

taken from a crab held in the Straits of Johore for 312 hours (13 days) following ecdysis (Table I). It can be inferred that this is near the maximum size for *O. cor* based on an examination of 190 barnacles from a series of pre-molt crabs and exuviae, which yielded a maximum capitular length of 4.576 mm (Table II). Based on carapace widths of 82.75 mm to 106.65 mm, these pre-molt crabs and exuviae corresponded to instars 15, 16, and 17, and were at least 50, 57, and 77 days respectively from the preceding instar (Ong, 1966; Hill, 1975). Clearly, near maximum barnacle size can be attained within a relatively short period of time (13 days) and the additional time between 13 and 50, 57, or 77 days does not result in much additional growth of *O. cor*. Thus, the two-week period which follows crab ecdysis is ideal for growth rate determinations. To estimate the rate of growth during this period, two methods were employed.

The first estimate utilized the large *O. cor* samples (122 and 113 adults) retrieved from 14 and 8 crabs exposed for 60–108 hours and 300–348 hours, respectively (Table I). These exposure periods are near the ends of the range. For the 60–108 hour period, the maximum capitular length observed was 1.859 mm, and for the 300–348 hour period it was 4.719 mm. Dividing the difference between the two capitular lengths (2.860 mm) by the difference in the exposure time of the two crabs hosting these barnacles (312 − 108 = 204 hours) yielded a growth rate estimate of 0.014 mm per hour, or 0.336 mm per day. This could be an underestimate of the growth rate if the barnacles did not attach very soon after the crab was placed in the Johore Straits, but not a significant overestimate unless the 4.719 mm barnacle is decidedly atypical. Although this does not appear to be the case, based on the survey of pre-molt crabs and exuviae listed in Table II, a second method of estimating growth rate minimizes the possibility of this type of error.

Two additional pieces of information needed for the second growth estimate calculation were obtained by examining the size distribution of all the metamorphosed barnacles within each exposure period. First this examination revealed that the smallest metamorphosed barnacle measured 0.572 mm. Second, it allowed determination of the probable length of the period between cyprid settlement and the conclusion of

TABLE II

The number and maximum capitular length of metamorphosed O. cor *obtained from gill pair number five of* S. serrata *pre-molt crabs and exuviae*

	Octolasmis cor	
	Number	Length (mm)
Pre-molt crabs		
1	52	3.575
2	2	3.861
3	6	4.004
4	22	3.575
5	29	3.146
	111	
Exuviae		
1	16	3.146
2	7	4.576
3	23	2.717
4	10	3.575
5	23	3.575
	79	

metamorphosis (and thus the base point in time at which the growth phase begins) as follows.

Two barnacles measuring 1.001 mm and 0.715 mm were found on two different crabs exposed 48 hours after ecdysis. No other metamorphosed barnacles were found within the gill chambers of the five crabs exposed 36–48 hours. Based on the minimum observed size of a metamorphosed barnacle, 0.572 mm, it appears that the 1.001 mm barnacle had already grown 0.429 mm following its metamorphosis. From the above growth rate estimate of 0.014 mm per hour, the 0.429 mm represents as much as 30.6 hours of growth. Thus the 1.001-mm barnacle found on the 48-hour crab may have metamorphosed as early as 17.4 hours (48–30.6) after attachment. However the paucity of metamorphosed barnacles (2) in the presence of numerous cyprids (106) in this exposure group of crabs argues that it would be more conservative, and realistic, to use 24 hours as the minimum time until metamorphosis and the beginning of the growth phase.

A second measure of growth was made by subtracting the minimum capitular length at metamorphosis (0.572 mm), from the capitular length of the barnacle whose growth rate was being estimated. This figure was divided by the number of hours the host crab was exposed minus 24 hours that allowed for cyprid attachment, metamorphosis, and onset of growth, to give the growth rate in mm per hour. The latter figure was rounded to three significant digits and multiplied by 24 to yield a mm per day growth rate. Table III presents these growth rate estimates for the three largest barnacles in each of the exposure intervals represented by at least five crabs.

Overall these growth rates ranged from 0.264 to 0.480 mm per day. Growth rates of the largest, second largest, and third largest barnacles ranged from 0.336 to 0.480 mm per day, 0.264 to 0.360 mm per day, and 0.264 to 0.408 mm per day, respectively. The amount of overlap among these estimates indicates that the growth rates of the largest barnacle in each interval were not substantially faster than the rates of the second and third largest barnacles. The growth rate of 0.336 mm per day calculated

TABLE III

Growth rate estimates for the three largest barnacles for each crab exposure interval represented by more than five crab hosts

Interval hours	Barnacle size (mm)	Hours of exposure	Growth rate mm/day
60–108	1.859	108	0.360
	1.716	108	0.336
	1.430	84	0.336
120–168	2.574	168	0.336
	2.145	156	0.288
	2.145	144	0.312
180–288	4.004	192	0.480
	3.289	204	0.360
	3.289	180	0.408
240–288	3.718	240	0.360
	3.718	288	0.264
	3.575	240	0.336
300–348	4.719	312	0.336
	3.718	312	0.264
	3.718	312	0.264

for the very largest barnacle in the study (4.719 mm) was not atypical. The mean, median, and mode of the 15 growth rate estimates are all 0.336 mm per day. In addition, the first method of calculating growth rate (comparing the largest barnacles of the 60–108 hour and 300–348 hour intervals) also resulted in an estimate of 0.336 mm per day.

DISCUSSION

The fastest growth rate, 0.480 mm per day, was attained by the largest barnacle in the 180–228 hour exposure interval (the second largest barnacle in the growth rate study overall). This rate is very close to the 0.5 mm per day reported for *Lepas hillii* Leach (Evans, 1958), growing under natural conditions on the side of a ship for one- and two-month periods at 24.2–26.1°C.

Our estimates support the contention that growth of *O. cor* is rapid, roughly a third to a half mm per day. For the largest barnacle (4.719 mm), 0.336 mm per day represents a daily growth increment of about 7% of its final capitular length.

The striking similarities among the growth estimates suggest that the techniques developed in this study allowed us to measure growth more precisely than was previously possible. Furthermore it appears that growth occurred under nearly optimal conditions. Freshly molted crabs provided a clean, uncrowded substrate, and the rich plankton flora and fauna of the Straits provided what was probably unlimited food for the barnacles. In a previous study (Jeffries *et al.*, 1982), 10 *Scylla serrata* were observed to host 1324 *O. cor* (an average of 132.4 per crab), plus 1337 individuals of another *Octolasmis* species, for a total average of 266.1 barnacles per crab. In this study the average number of metamorphosed *O. cor* per crab was 11.9; adding in the average number of individuals of other species, the total average number of individual barnacles on each crab was 24.5.

The estimate made in this study of the time between attachment of the *O. cor* cyprid larvae until metamorphosis, 24 hours at temperatures of 28.4–30.8°C, compares favorably with the reported time of 20 to 72 hours at 24–29°C for *O. mulleri* reared in the laboratory (Lang, 1976). Another genus of barnacle, *Balanus amphitrite variegatus,* required only 37 minutes for cyprid attachment through metamorphosis, at an unspecified temperature in the laboratory (Daniel, 1958).

In contrast to the high incidence of gravid individuals among the *O. cor* collected from pre-molt crabs and exuviae, not one of the 837 *O. cor* examined in this growth study was gravid, although many were sexually mature. This suggests several interesting possibilities regarding the growth strategy of *O. cor,* which we can now begin to explore.

Attainment of sexual maturity by *O. cor* within two weeks on a host crab which spends 50–77 days between molts has a significant advantage: it allows for increased egg production, through the increased numbers of egg clutches that are possible over a greater period of time. In addition, previous work on a related barnacle, *O. mulleri,* on the blue crab *Callinectes sapidus,* demonstrated a strong positive correlation between capitular size and brood size (Jeffries and Voris, 1983).

A second advantage of rapid growth and attainment of sexual maturity, is that the number of available hosts is effectively increased: The ninth instar of *Scylla serrata* is a minimum of 15 days from the previous instar according to Ong (1966). Thus the compression of *O. cor* growth to sexual maturity into a short period of about two weeks allows it to utilize smaller (and more numerous) hosts, thereby enabling it to further increase its usable substrate.

We suspect that the barnacles are attuned, perhaps hormonally, to the physiological state of the crabs, allowing them to direct their resources toward growth during the two weeks immediately following crab ecdysis.

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THE SHADOW RESPONSE OF A HYDROMEDUSAN (*POLYORCHIS PENICILLATUS*): BEHAVIORAL MECHANISMS CONTROLLING DIEL AND ONTOGENIC VERTICAL MIGRATION

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ABSTRACT

A variety of photic stimuli were presented to the hydromedusan *Polyorchis penicillatus* under controlled conditions to characterize the photic responses of various sizes of *P. penicillatus* and to determine the role of these responses in its behavior. "Treadmill" experiments showed that for all but very small hydromedusae, swimming frequencies at different constant light intensities did not differ. Swimming frequency of *P. penicillatus* was, however, directly proportional to rates of decrease in light intensity. Slowly increasing light intensity caused an inhibition of swimming and "crumpling." Rapid, 100% shadows of various absolute magnitudes usually caused only a single swimming contraction. The maximal response to rapid shadows of monochromatic light occurred around 450–550 nm. These results suggest that the shadow response of *P. penicillatus* does not function in predator avoidance, but more likely contributes to nighttime upward movement in the water column. The inhibition of swimming during increasing light intensity may initiate dawn sinking. Most of the photic responses of *P. penicillatus* show size- (age) related differences which may result in ontogenic changes in distribution and feeding behavior.

INTRODUCTION

Many hydromedusae show distinct behavioral responses to changing light conditions. Some species of hydromedusae respond to rapidly decreasing light intensities with a few rapid swimming contractions. This behavior has been generally referred to as an "off response" (Singla, 1974), a "shadow response" (Tamasige and Yamaguchi, 1967), or a "shadow reflex" (Kikuchi, 1947). Although the function of this stereotypic behavior has never been demonstrated, traditionally it has been considered a predator avoidance mechanism. Many hydromedusae also respond to changes in light intensity as evidenced by their diel vertical migrations (Russell, 1925; Moreira, 1973; Mills, 1982). A few of these species, such as *Bougainvillia principis, Gonionemus sp., Polyorchis penicillatus, P. karafutoensis, Spirocodon saltatrix,* and *Stomotoca atra,* that have been shown to have a shadow response (Murbach, 1909; Kikuchi, 1947; Hisada, 1956; Tamasige and Yamaguchi, 1967; Singla, 1974; Mackie, 1975; Anderson and Mackie, 1977) also make distinct diel vertical migrations (Mills, 1982, 1983; Arkett, 1984). Even though rapidly changing light intensity at dawn and dusk is generally considered one of the most important cues used by zooplankters to initiate and regulate diel vertical migration (McNaught and Hasler, 1964; Ringelberg, 1964; Forward, 1976a; Buchanon and Haney, 1980; Stearns and Forward, 1984), the possibility of a causative relationship between the shadow response of hydromedusae and their diel vertical migration has not been considered previously.

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Changing photic stimuli have been shown to alter the swimming activity of the hydromedusan, *Polyorchis penicillatus* (Anderson and Mackie, 1977). Anderson and Mackie (1977) showed that *P. penicillatus* responds to shadows with a burst of action potentials from the swimming motor neurons (SMNs—Spencer, 1979, 1981). Each action potential in the burst produces a contraction in the swimming muscles and hence one swimming contraction. This response to shadows is lost if the ocelli are removed, suggesting that the radially arranged ocelli are essential to the detection of shadows (Anderson and Mackie, 1977). Additionally, Anderson and Mackie (1977) found that the SMNs were directly photosensitive, that is, the membrane potential and the frequency of action potentials (and thus swimming frequency) of the SMNs were directly related to the ambient light intensity. From this finding, Anderson and Mackie (1977) suggested that diel vertical migration of medusae may be controlled by the effects of ambient light intensity on photosensitive swimming motor neurons. Thus, one might predict that *P. penicillatus* would show reverse diel vertical migration (*i.e.*, swimming up into the water column during daylight and sinking at night). However, Mills (1983) and Arkett (1984) have shown that *P. penicillatus* is a diel vertical migrator, occurring near the bottom during daytime and up in the water column at night. To reconcile this apparent contradiction, I have characterized the photic responses of various sizes, and thus ages, of *P. penicillatus*. Medusae were exposed to a variety of photic stimuli, simulating those produced by potential predators and those found during changing light conditions at various times of day, to determine the functions of the photic responses.

Although several laboratory studies have previously examined the photic behavior of medusae swimming freely in aquaria (Murbach, 1909; Mackie *et al.*, 1981; Mills, 1983), at least two aspects of these studies may bias their results. First, as medusae confined in aquaria collide with the walls, their swimming activity is altered. Swimming may be either inhibited by "crumpling" (Hyman, 1940) or increased by excitation of the tentacles upon contact with the tank walls. Secondly, with free-swimming medusae, it is difficult to control the lighting regime because the position of the medusa in the water column and the orientation of its ocelli with regard to light sources is constantly changing. I have eliminated these problems in this study by designing a "treadmill" which allows tethered swimming of medusae, eliminates wall effects, and maintains a constant orientation of ocelli to lighting.

MATERIALS AND METHODS

Description of treadmill and lighting

Medusae of various sizes were suspended on the treadmill by fine (1 mm diameter) glass tubing (Fig. 1). Some very small individuals required suspension by finer wire. The tubing pierced the exumbrellar epithelial layer of the bell and passed through the thick mesogloea at the bell apex. Neither swimming musculature on the subumbrellar surface nor any nervous tissue was disrupted. Tubing ends rested on the sides of a 21 × 21 × 18 cm plexiglass tank and thus maintained the bell margin bearing the ocelli horizontal. Tentacles could not touch the sides or bottom of the tank. In this position, medusae could perform much of their behavior (*e.g.*, swimming, crumpling, feeding) without moving around the tank.

The light source for experiments was a Volpi AG Intralux fiber optic system equipped with a halogen lamp. Absolute light intensities were measured with a Licor LI-185 Quantum Sensor. Light intensity was altered by opening and closing an iris diaphragm located between the light source and fiber optic and by adjusting distances

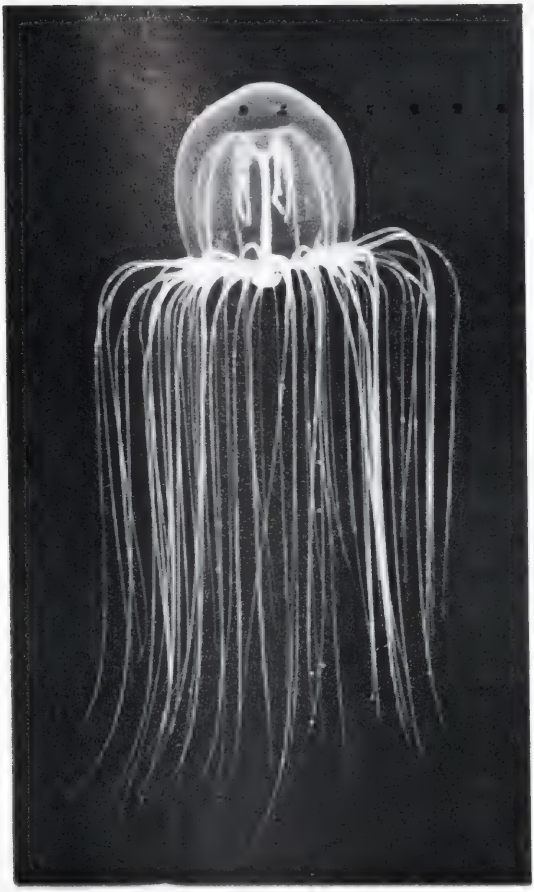

FIGURE 1. Side view of *P. penicillatus* suspended on the "treadmill." Medusae were completely immersed and free from contact with sides and bottom of the tank. Individuals often assumed a typical "sink-fishing" posture shown here and could swim freely. Fiber optic lighting was suspended directly above the medusa. Distance between the marks on glass tubing is 1 cm. Bell height (measured from apex of bell to bell margin) was used as a measure of medusan size.

between the fiber optic head and bell margin. Specifics of lighting conditions for each experiment are given in their respective sections. All experiments were conducted in a darkened room, the light intensity of which was below that detectable by the Licor light meter (less than 0.1 microeinsteins/$m^2 \cdot s$). Seawater temperature in the treadmill ranged from 10–13°C. Treadmill seawater was changed after each medusa.

Individuals of *P. penicillatus* were collected by divers from Bamfield Inlet and Pachena Bay, Bamfield, British Columbia, Canada. Medusae were kept unfed in running seawater and were used within 3–4 days. All animals were kept under natural photoperiod prior to the experiments.

Medusa size/spontaneous swimming-frequency

In the field, *P. penicillatus* alternates from "maintenance swimming" at frequencies of about 5–15 swims per min (depending on size) to spontaneous extended swimming bouts at higher frequencies (Arkett, 1984). This experiment was designed to examine the size/swimming-frequency relationship during these extended swimming bouts. Seventeen medusae of various sizes were placed singly on the treadmill and allowed to acclimate for 15 minutes. This time period was sufficient for medusae to relax tentacles and attain a sink-fishing posture. Under constant light conditions (ceiling fluorescent lights), the total number of swimming contractions during spontaneous extended swimming bouts was counted. Three to five separate bouts were observed for each medusa. The mean number of swimming contractions per minute was plotted as a function of bell height.

Swimming frequency/different constant light intensities

In these experiments, *P. penicillatus* was illuminated with two different light intensities, representative of those found in daytime surface or bottom waters of Bamfield Inlet, to examine photokinetic effects on swimming frequency. Twenty-nine medusae were placed singly on the treadmill and they were allowed to acclimate for 15 minutes at LOW (9.7 microeinsteins/$m^2 \cdot s$) light intensity. At the start of the experiment, the diaphragm was opened over 15 seconds to create the HIGH (280.7 microeinsteins/$m^2 \cdot s$) light intensity. The number of swimming contractions was counted for a total of 15 minutes (beginning 15 seconds after opening the diaphragm) with the first 2 minutes divided into 15 s intervals (8 time periods). After 2 minutes, the number of swimming contractions was counted for each of the remaining thirteen 1 minute intervals. At the end of the 15 minute trial period, the diaphragm was closed over 15 s to create the LOW light intensity and swimming contractions were counted as before. This procedure was followed by one more HIGH light and one more LOW light intensity trial, totalling two LOW and two HIGH light intensity trials per medusa. A two-way ANOVA (Sokal and Rohlf, 1969) was performed on log (x + 1) transformed values (x = calculated number of swimming contractions per s) comparing differences in swimming frequency at the 2 light intensities and 21 time periods. The total number of individuals was separated into three arbitrary size classes, those with bell heights less than 1 cm, 1–2 cm, and greater than 2.0 cm. Since replicate trials for one medusa were not independent in this and the remaining experiments, the degrees of freedom for all statistical tests are based on the number of individual medusae and not on the total number of trials.

Shadow response/rapid 100% shadows

Rapid shadows of different absolute light intensity change, but of the same relative change, were presented to determine whether the shadow response was affected by the absolute light intensity change. Nine medusae of various sizes were placed singly on the treadmill and were allowed to acclimate in the dark for 15 minutes. Four light intensities (280.7, 65.8, 9.7, 2.4 microeinsteins/$m^2 \cdot s$) were separately presented for 2 minutes and then a rapid, OFF-ON 100% shadow was made by passing a card between the fiber optic head and the medusa. The duration of the OFF period was approximately 0.5 s. The percent change in light intensity was determined by $(I_0 - I_1)/I_0$ where I_0 = initial absolute light intensity and I_1 = light intensity after 0.5 s (in this case approximately 0 microeinsteins/$m^2 \cdot s$). During the two minute pre-shadow period, the number of swimming contractions per minute was counted. After the shadow, the number of swimming contractions in five seconds was recorded. The shadow response, as indicated by the number of swimming contractions per minute following the stimulus, was determined by subtracting the pre-shadow swimming frequency from the post-shadow swimming frequency. Four trials per individual were made at each of the four light intensities in random order. Comparisons of the mean swimming frequency [after log (x + 1) transform] of the shadow response at the four different absolute light intensities were made by a one-way ANOVA (Sokal and Rohlf, 1969).

Shadow response/spectral sensitivity

The spectral sensitivity of the shadow response was determined in experiments similar to the preceding ones, but with monochromatic light. Seventeen medusae of various sizes were placed singly on the treadmill and they were allowed to acclimate in the dark for 15 minutes. Medusae were then exposed to 25 nm increments of monochromatic light ranging from 425 to 700 nm. Monochromatic light was produced by shining the fiber optic light source into a Bausch and Lomb Monochrometer (band pass width 19.2 μm; dispersion 6.4 μm/mm; first order range 350–800 nm). All light intensities were adjusted to 0.7 microeinsteins/m$^2 \cdot$ s, the greatest light intensity attainable for all wavelengths. Medusae were illuminated with monochromatic light for 2 minutes and then given a rapid (0.5 s duration), OFF-ON 100% shadow as described previously. If a swimming contraction followed the shadow within 1 s, I considered that the individual was able to respond to that wavelength and assigned a value of 1 to that trial. Additional swimming contractions after 1 s were judged to be not due to the shadow and were not counted. If the medusa did not respond, I assigned a value of 0. Four such trials for each individual were conducted at each wavelength increment in random order. The spectral sensitivity of the shadow response is reported as a percent of the maximum possible number of responses for each wavelength increment.

Shadow response/continuous changes in light intensity

Continuous decreasing and increasing light intensities were presented to *P. penicillatus* to determine how its response to continuous changes in light intensity differs from its response to rapid OFF-ON shadows. Eleven medusae of various sizes were placed singly on the treadmill and allowed to acclimate in the dark for 15 minutes. Medusae were then presented with four different rates of light intensity change and the observed swimming frequency was recorded. Changing light conditions were produced by manually opening and closing an iris diaphragm located between the fiber optic and the light source. The light intensity at the bell margin ranged from 280.7 microeinsteins/m $\cdot$ s (diaphragm fully open, HIGH) to 9.7 microeinsteins/m$^2 \cdot$ s (LOW). The light intensity changes produced by opening and closing the diaphragm were not linear (Fig. 2). Changes in diaphragm position (HIGH to LOW and LOW to HIGH) were made over four time periods (1, 15, 30, 60 s) and corresponded to the log of the mean percent change in light intensity per s ($\Delta I_{\bar{x}}$) of −0.015, −0.914, −1.123, −1.344, respectively. Although the rates at which the diaphragm was opened and closed were identical for both increasing and decreasing light intensity trials, these values were calculated from decreasing light intensity only.

Medusae were exposed to LOW light for 15 minutes after which the diaphragm was opened to HIGH, followed by a change from HIGH to LOW. A 2-minute interval separated each change and 5 such increasing and 5 decreasing light intensity trials were given to each individual for each of the four rates of change. The total number of swimming contractions during these periods of light intensity change was counted and the mean number of swimming contractions per minute was calculated. Medusae were divided into two arbitrary size classes (<2.0 cm and >2.0 cm bell heights). Com-

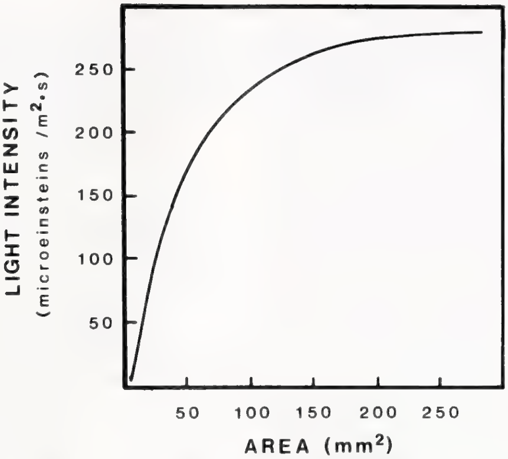

FIGURE 2. Stimulus curve for continuous changes in light intensity showing the area of iris diaphragm aperture and light intensity of the fiber optic at a distance of 10 cm. A logarithmic curve ($Y = -121.17 + 73.62 \ln x$, $r = 0.977$) has been fitted to the measured values. The curve was divided into 1, 15, 30, 60 second intervals and the absolute light intensity change per s was calculated for each of the four time intervals. The mean rate of percent change in light intensity per s ($\Delta I_{\bar{x}}$) was determined for each of the four time intervals by $(I_0 - I_1)/I_0$ where I_0 = initial absolute light intensity and I_1 is the light intensity after 1 s.

parisons between the mean swimming frequency at each rate of light intensity change were made by one-way ANOVA (Sokal and Rohlf, 1969).

RESULTS

Medusa size/spontaneous swimming frequency

Individual swimming bout durations ranged from as short as fifteen seconds to longer than five minutes. Swimming frequency during each bout was usually very regular. Quiescent periods of sink-fishing or an occasional crumple separated bouts. The mean swimming frequency of *P. penicillatus* during extended swimming bouts increased exponentially with decreasing bell height (Fig. 3). In the field, *P. penicillatus* usually swims in an arc at frequencies close to those seen in Figure 3 (Arkett, 1984).

Swimming frequency/different constant light intensities

Small medusae swam at a greater frequency under constant HIGH light intensity than under LOW light intensity; large medusae swam at nearly the same frequency regardless of the light intensity (Fig. 4). A two-way ANOVA comparing the mean number of swimming contractions per second at two light intensities and 21 time increments showed that medusae with bell heights less than 1.0 cm swam at significantly ($0.05 > P > 0.01$) greater frequency under HIGH than under LOW light intensity. For the two groups of medusae with bell heights greater than 1.0 cm, there was no significant ($P > 0.05$) difference in swimming frequency under the two light intensities. Although the group of smallest medusae showed an initial reduction in swimming frequency under HIGH light intensity, suggesting some adaptation to the light intensity, none of the groups showed a significant ($P > 0.05$) difference in swimming frequency over the 15 minute period.

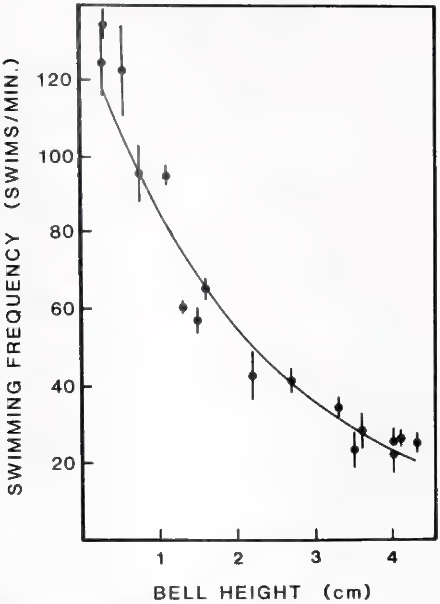

FIGURE 3. Mean (±1 SE) number of swimming contractions per minute of various sizes of *P. penicillatus* observed during spontaneous extended swimming bouts on the treadmill. An exponential curve ($Y = 132.65\ e^{-0.43x}$; $r = 0.96$) has been fitted to the mean swimming frequencies. The smallest individuals used were early eight and sixteen tentacle stages. Each mean represents a single individual.

Shadow response/rapid 100% shadows

Medusae of all sizes tested (0.9–3.3 cm bell height) usually responded to rapid 100% shadows with one rapid swimming contraction, regardless of the absolute magnitude of the light intensity change (Table I). During the two minute pre-shadow period, most of the individuals did not swim and remained nearly motionless in the sink-fishing posture. Immediately after the brief shadow was presented, nearly all of these medusae responded with one swimming contraction. This single swimming contraction did not lead to extended swimming bouts and medusae returned to their sink-fishing posture. For those medusae that were swimming during the pre-shadow period, the response to the shadow was one additional swimming contraction in its pre-shadow swimming frequency. There were no size-related differences in the response to the rapid shadows.

Shadow response/spectral sensitivity

All medusae consistently responded to shadows of monochromatic light between 450–575 nm (Fig. 5). Small medusae responded to a broad range of wavelengths with a peak response at 450 nm while the response range of larger medusae was compressed (failing to respond at 675 nm) and the peak response shifted to a slightly longer (550 nm) wavelength. These peaks and spectral distribution of the shadow response is similar to the spectral response of the electroretinogram (ERG) of *P. penicillatus* found by Weber (1982). Smaller individuals were generally more responsive to all wavelengths as indicated by the higher overall percentage of responses.

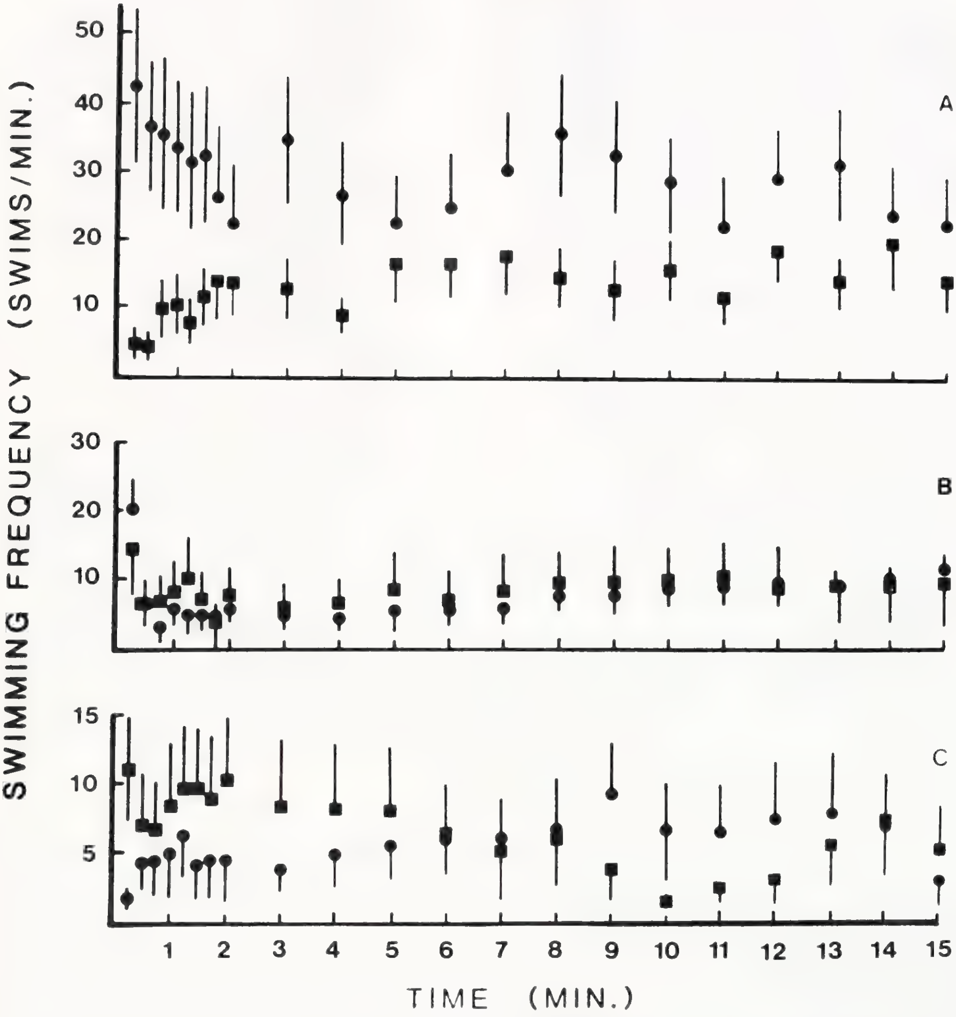

FIGURE 4. Mean (±1 SE) number of swimming contractions per minute by *P. penicillatus* on the treadmill over 15 min periods at HIGH (● 280.7 microeinsteins/$m^2 \cdot s$) and LOW (■ 9.7 microeinsteins/$m^2 \cdot s$) light intensity. (A) Swimming frequency of individuals with bell heights less than 1.0 cm (range 0.3–0.7 cm, n = 24, 12 individuals); (B) swimming frequency of individuals with bell heights between 1–2 cm (range 1.2–1.8 cm, n = 16, 8 individuals); (C) swimming frequency of individuals with bell heights greater than 2.0 cm (range 2.1–3.4 cm, n = 18, 9 individuals). Notice the size class differences in overall swimming frequency with smaller medusae swimming at a much higher frequency than larger individuals. *N.B.*, the means and SE presented here are from raw data and are not antilogarithms of the log (x + 1) transformed data.

Shadow response/continuous changes in light intensity

The swimming frequency of *P. penicillatus* decreased markedly as the rate of decrease in light intensity decreased (Fig. 6). Medusae swam almost continuously throughout the period of decreasing light intensity. Thus, the greatest total number of swimming contractions occurred during the slowest rate of decrease in light intensity

TABLE I

Mean (±1 SE, $n = 36$) number of swimming contractions in the 5 s period after a rapid 100% shadow of four different absolute light intensity changes. Comparisons of the mean shadow response frequency at four absolute light intensity changes by one-way ANOVA showed no significant ($P > 0.05$) difference in the responses

Light intensity (microeinsteins/$m^2 \cdot s$)			
2.4	9.2	65.8	280.7
0.84 (0.08)	1.08 (0.09)	1.01 (0.13)	0.98 (0.02)

(Table II). The highest swimming frequency was observed during the most rapid light intensity reductions, but the total number of swimming contractions in the response was small (Table II). Smaller medusae showed a greater overall swimming frequency and were more responsive to rapid shadows (*i.e.*, exhibited greater swimming frequency than larger medusae). For slower shadows, however, larger medusae showed a greater swimming frequency.

During increasing light intensity, medusae rarely swam and often crumpled, resulting in marked differences in swimming frequencies from those seen during decreasing light intensity (Fig. 6). Crumpling behavior was most obvious during the slower light intensity increases and consisted of progressive tentacle contractions, bell margin involutions, and radial muscle contractions. Once fully crumpled, medusae often remained so for several minutes. If a medusa was in the crumpled position at the start of a successive decreasing light intensity trial, it usually relaxed the severe contractions, responded to the shadow with a swimming contraction, and began to swim at a frequency proportional to the rate of light intensity decrease. There were

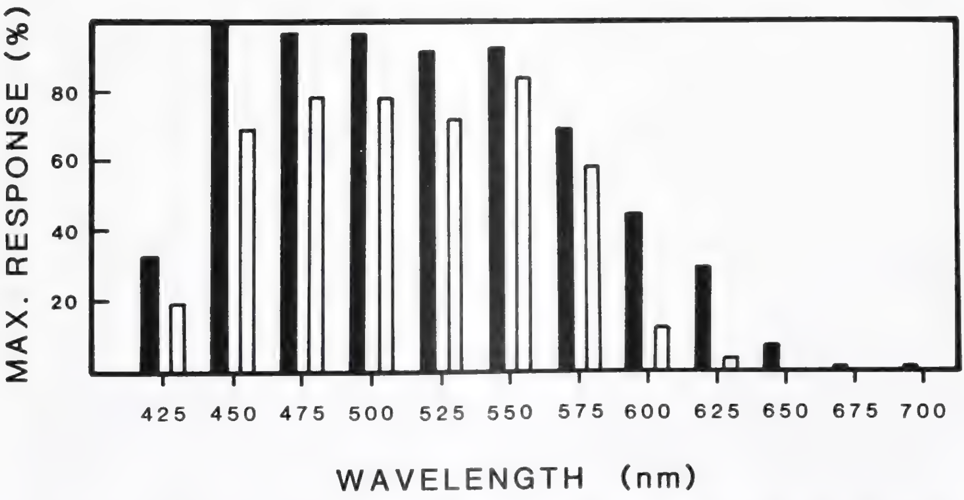

FIGURE 5. Percent of the maximum possible number of shadow responses to rapid shadows of monochromatic light. Solid columns indicate individuals with bell heights less than 2.0 cm (10 individuals), open columns indicate individuals with bell heights greater than 2.0 cm (7 individuals). The light intensity has been adjusted to 0.7 microeinsteins/$m^2 \cdot s$ for each wavelength.

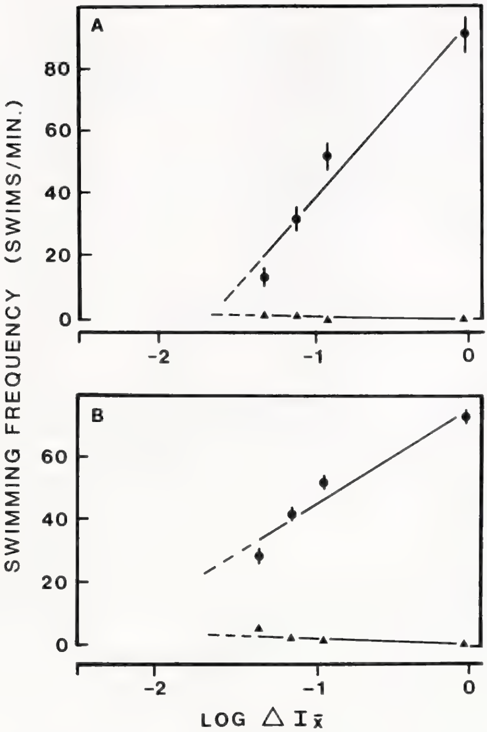

FIGURE 6. Mean (±1 SE) number of swimming contractions per minute by *P. penicillatus* in response to four mean rates of percent change in light intensity per s ($\Delta I_{\bar{x}}$). (A) Responses of medusae with bell heights less than 2.0 cm (range 0.7–1.9 cm, 6 individuals). There was a significant ($P < 0.001$) difference between the four mean swimming frequencies and a significant ($0.05 > P > 0.01$) linear regression ($Y = 94.42 + 56.77 X$) for decreasing (●) light intensity. No significant ($P > 0.05$) difference between the mean swimming frequencies or regression ($Y = -0.16 - 0.52 X$) for increasing (▲) light intensity. (B) Responses of medusae with bell heights greater than 2.0 cm (range 2.4–2.8 cm, 5 individuals). There was a significant ($P < 0.001$) difference between the four mean swimming frequencies and a significant ($0.05 > P > 0.01$) linear regression ($Y = 75.40 + 31.09 X$) for decreasing light intensity. For increasing light intensity, there was no significant ($P > 0.05$) difference between the four means. Linear regression ($Y = 0.43 - 2.49 X$) is not significant ($P > 0.05$). For both size classes, swimming frequencies at the greatest rate of percentage decrease is probably close to the maximum swimming frequency. Dashed lines show predicted swimming frequencies for other rates of light intensity changes.

no obvious differences in the response to increasing light intensity between the different sizes of *P. penicillatus* (Fig. 6); nearly all individuals, regardless of size, showed at least partial crumpling. Similar results were observed when the absolute light intensity for

TABLE II

Mean (±1 SE) total number of swimming contractions duringn four different rates of percentage change in light intensity ($\Delta I_{\bar{x}}$). Values are for decreasing light intensity only. (1) For individuals with bell heights less than 2.0 cm, n = 30. (2) For individuals with bell heights greater than 2.0 cm, n = 25

	LOG $\Delta I_{\bar{x}}$			
	−0.015	−0.914	−1.123	−1.344
(1)	1.51 (0.12)	12.73 (1.17)	15.53 (2.02)	12.17 (2.83)
(2)	1.22 (0.03)	13.08 (0.52)	21.00 (1.03)	28.76 (2.73)

HIGH was 9.4 microeinsteins/$m^2 \cdot s$ and LOW was 0.2 microeinsteins/$m^2 \cdot s$ at the same rates of percent change in light intensity.

DISCUSSION

Results from this study indicate that predator avoidance is an unlikely function of the shadow response of *P. penicillatus,* although the shadow response of some medusae (Singla, 1974; Anderson and Mackie, 1977) and other animals (Gwilliam, 1963, 1965; Forward, 1976b; Forward, 1977; Moore and Cobb, 1985) traditionally has been considered to function as such. A typical shadow generated by some cruising predator, such as a fish, might be a rapid OFF-ON shadow of a duration similar to that used in the experiments shown in Table I, although a predator-generated shadow would probably be less than a 100% decrease in light intensity. *P. penicillatus* responds to such rapid shadows with 1–2 additional swimming contractions (Table I). These swimming contractions would not propel the medusa far or fast enough to escape rapidly swimming predators because distances travelled are negligible until the medusa reaches maximum velocity, which usually occurs after 1–2 swimming contractions (Gladfelter, 1972; Daniel, 1983). Furthermore, the subsequent increase in light intensity following the shadow would inhibit further swimming contractions (Table I, Fig. 6). Some medusae eat other medusae (Plotnikova, 1961; Zelikman, 1969; Fraser, 1969; Arai, 1980) and predatory medusae might produce slow, long duration shadows causing extended upward swimming (Fig. 6). These movements might result in escape from these relatively slow moving predators. However, even if *P. penicillatus* does respond to predator-generated shadows, it seems unlikely that the resultant movement would yield any advantage to the medusae. Rather, because the potential predator creating the shadow would necessarily be positioned above the medusa (ocelli are oriented upward on the abaxial surface of each tentacle base), the upward swimming in response to such a shadow would only increase the probability of detection and capture as the medusa moves closer to the predator. Furthermore, known predators of *P. penicillatus* in Bamfield Inlet (pers. obs.) are benthic and would not produce shadows. *P. penicillatus* is occasionally found in the tentacles of and in the pharynx of sea anemones (*e.g., Urticina* sp., *Pachycerianthus* sp.) and in the chelipeds of crabs (*e.g., Cancer productus*). These benthic predators capture *P. penicillatus* because this hydromedusan spends much of its time near the bottom (Arkett, 1984).

The swimming frequency of *P. penicillatus* observed under constant light (Fig. 4) is similar to the "maintenance swimming" frequency observed in the field (Arkett, 1984). Constant light intensities of different absolute magnitudes do not cause differences in these swimming frequencies, at least for larger medusae. Net vertical movement during maintenance swimming is neglible because swimming contractions and upward movements are separated by short sinking periods. However, any increase or decrease in this swimming frequency over extended periods of time should result in a change in the position in the water column. Marked differences in maintenance swimming frequency have been observed in the field during extended swimming bouts, as *P. penicillatus* usually swims in an arc at frequencies close to those seen in Figure 3 (Arkett, 1984). Even though the swimming frequency and the duration of these bouts are sufficient to displace the medusae appreciable distances, the bouts are transient, apparently spontaneous, and are probably not a direct result of changing light conditions. That swimming frequency for large medusae does not increase with constant higher light intensities would explain why *P. penicillatus* is not found up in the water column during the daytime (Mills, 1983; Arkett, 1984). The lack of a direct relationship between swimming frequency and ambient light intensity are contrary to the findings of Anderson and Mackie (1977) and are contrary to predictions of swimming activity and hence water column position. However, there is more recent electrophysiological evidence that the SMNs are not directly photosensitive (Arkett and Spencer, in prep.). Therefore, swimming frequency and water column position are not a direct function of ambient light intensity.

Slow, continuous reductions in light intensity cause nearly continuous swimming of *P. penicillatus* at frequencies above maintenance swimming frequency and for extended periods (Fig. 6). These recurring shadow responses should displace individuals significant distances and result in upward movements. Whether recurring shadow responses in response to continuously decreasing light intensity contribute to the diel vertical migration of *P. penicillatus* depends upon the rate of change in light intensity under field conditions. In one of the few field studies that has recorded rates of change in light intensity, Munz and McFarland (1973) showed that the greatest rate of light intensity decrease does not occur until after sunset and for about 40 minutes after that time. During this period the mean rate of percent decrease in light intensity (over a selected period) was approximately 88% in 7 min (=0.21%/s; =0.0021/s, log x = −2.67) (Munz and McFarland, 1973). Stearns and Forward's (1984) study in estuarine coastal waters showed that the mean rate of percent decrease in light intensity (over a single 10 min interval from between sunset and about 50 min after sunset) was approximately 0.16%/s. Attempts were made to measure the rate of percent change in light intensity every 10 minutes before and during sunset in Bamfield Inlet on several days in May 1983. The greatest rate of decrease in light intensity that could be recorded before light intensity fell below the sensitivity of the light meter was 90% in 10 minutes (=0.15%/s). These rates are about 10 times slower than the slowest rates that could be produced in my experiments (Fig. 6). However, the predicted swimming frequency of large medusae (greater than 2.0 cm bell height, Fig. 6) for such rates of change in light intensity are close to, or slightly greater than, those found during constant light intensity experiments (Fig. 4) and the maintenance swimming frequency found in the field (5–10 swims/min; Arkett, 1984). This small increase in swimming frequency may then contribute to the initiation of upward movement at sunset and the diel vertical migration of *P. penicillatus.* However, for small medusae, (less than 2.0 cm bell height, Fig. 6) predicted swimming frequency for rates of decrease in light intensity at sunset is well below that of their maintenance swimming frequency (10–15 swims/min; Arkett, 1984) and their swimming frequency in constant light intensities (Fig. 4). It thus remains unclear whether slow reductions in light intensity initiate diel vertical movement in young medusae.

The timing of the initiation of the upward movement in most plankters appears to be governed by the threshold rate of light intensity decrease necessary to elicit the shadow response. The swimming reaction of *Daphnia magna* is not initiated until the rate of percent decrease reaches 0.17%/s, even though the rate of decrease in light intensity at dusk ranged from 0.13%/s to 0.24%/s (Ringelberg, 1964). Stearns and Forward (1984) also found that the copepod *Acartia tonsa* did not move upward in the water column in appreciable numbers until about 30–40 minutes after sunset. Pre-sunset rates of decrease in light intensity (approximately 20% over a 3 min interval) did not cause pronounced upward movement (Stearns and Forward, 1984). Munz and McFarland (1973) found that the "quiet period" in coral reef fishes also occurs only during the most rapid changes in light intensity: after sunset. Although the threshold rate of change in light intensity for the shadow response in *P. penicillatus* was not determined, predicted swimming frequencies (Fig. 6) at rates comparable to those of Stearns and Forward (1984) and Munz and McFarland (1973) and those recorded near the bottom of Bamfield Inlet 10–30 min prior to sunset (*e.g.*, 0.03%–0.05%/s) would be well below maintenance swimming frequency and below the swimming frequencies found at constant light intensities (Fig. 4). Thus, only when light intensity is rapidly decreasing near and after sunset (Rosenberg, 1966) would recurring shadow responses be elicited and swimming frequencies above maintenance swimming be reached. Only then could net upward movement result. Slower rates of decrease in light intensity, which occur late in the day and early evening, would probably not cause recurring shadow responses and net upward movement. This reasoning may explain why *P. penicillatus* begins to appear off the bottom and in the water column only after sunset and does not occur in large numbers up in the water column until several hours later (Mills, 1983; Arkett, 1984). Recurring shadow responses could, however, only initiate upward movement at sunset; they cannot explain the sustained nighttime position up in the water column. Sweatt and Forward (1985) found that the chaetognath *Sagitta hispida* continues upward swimming even in darkness. Although this continuous upward swimming may account for the sustained shallower water column position of *S. hispida,* this explanation does not appear to hold for *P. penicillatus.* Swimming in response to decreasing light intensity usually stops as soon as the rate of change in light intensity goes to zero. It is unknown how *P. penicillatus* maintains the nighttime water column position because it shows neither diel changes in ionic concentration (Mills and Vogt, 1984), which may contribute to buoyancy changes, nor intrinsic circadian rhythms (Mills, 1983).

The marked decrease in swimming frequency during increasing light intensity (Fig. 6) may explain why *P. penicillatus* moves downward and aggregates near the bottom just after dawn (Mills, 1983; Arkett, 1984). At rates of increasing light intensity representative of field conditions, the predicted swimming frequency falls well below that of maintenance swimming (Fig. 6). This alone should account for a net downward movement near dawn since *P. penicillatus* is usually negatively buoyant (Mills, 1984), sinking at rates of between 15–35 cm per min (depending on its tentacle extension, Mills, 1981). Even more important than a reduction in swimming frequency is the fact that during increasing light intensity, severe crumpling occurs. This behavior is common to many medusae and it is often a response to noxious stimuli. Because this behavior increases normal sinking rates, it has usually been considered an escape mechanism; rapidly sinking to avoid predators (Hyman, 1940). However, the progressive, sustained crumpling and resultant rapid passive sinking in response to increasing light intensity suggests that this behavior causes dawn downward movement. The rate of light intensity increase necessary to elicit the inhibition of swimming also appears to ensure the timing of the pre-dawn sinking of *P. penicillatus* (Mills, 1983; Arkett, 1984). As the rate of percent increase in light intensity is most rapid before sunrise (McNaught and Hasler, 1964), the initiation of sinking due to the inhibition of swimming should occur just before sunrise. Because the sustained crumpling often lasts the duration of the increasing light intensity period (Fig. 6), medusae should be found in deeper water during and just after sunrise.

Passive sinking in response to increases in light intensity at dawn also appears to be a common behavior among other zooplankters (*e.g.,* cladocerans, copepods, brachyuran larvae) (Daan and Ringelberg, 1969; Forward *et al.,* 1984; Stearns and Forward, 1984; Sulkin, 1984). This response, in addition to causing dawn sinking, has also been suggested to regulate daytime depths. Light intensity increases, encountered by plankters during the day, act as a barrier against upward movements (Pearre, 1973; Forward *et al.,* 1984). The deep daytime position of *P. penicillatus* is probably maintained similarly. This explanation is supported by the observation that the continuous swimming, observed during slow decreasing light intensity, can be stopped by interposed light intensity increases. Even if rapid shadows of sufficient magnitude to elicit a response do occur during the daytime, due to waves, clouds, or eel grass, net upward movement would not result because subsequent increases in light intensity would inhibit swimming. However, Ohtsu (1983) has suggested another mechanism by which the deep daytime position of the hydromedusan *Spirocodon saltatrix* may be regulated. He found that ultraviolet (UV, 350 nm) light hyperpolarizes the swimming motor neurons and thereby inhibits swimming. Ohtsu (1983) suggested that as medusae swim upward in the water column toward the surface, they encounter increasingly greater intensities of UV light, which should inhibit swimming and eventually cause sinking. It is conceivable that Ohtsu's explanation of sinking upon approach to surface waters may apply to *P. penicillatus,* but because the effect of UV light was not examined with *P. penicillatus,* no conclusions can be made at this time. However, this explanation seems unlikely because many young medusae are often found in surface waters (see below).

In addition to changes in light intensity the spectral shift in light penetrating the water during sunrise and sunset may influence the shadow response. During sunset, the underwater spectrum tends to shift toward shorter wavelengths (450–500 nm, blue) (Munz and McFarland, 1973). However, the large amount of detrital matter or "yellow substance" in coastal waters (Jerlov, 1966) would tend to absorb blue light, shifting maximum transmission to green (500–550 nm). The spectral quality of the water in Bamfield Inlet is unknown, but the large amount of detritus present probably shifts maximum transmission to green, which is close to the maximal shadow response for larger medusae (Fig. 5). It is unknown whether spectral shifts at sunset and sunrise alter the sensitivity of the response to continuous changes in light intensity.

Ontogenic changes in photosensitivity in planktonic invertebrates are common (Thorson, 1964; Pearre, 1979) and these changes affect the vertical distribution and feeding behavior of later stages and adults (Pearre, 1973; Buchanan and Haney, 1980; Forward and Costlow, 1974; Cronin, 1982). Differences in the responses of various sizes of medusae to light stimuli found in this study suggest that *P. penicillatus* also undergoes ontogenic changes in its photic behavior. Smaller medusae showed: (1) a distinct photokinetic effect with swimming frequency (Fig. 4); (2) a slightly broader spectral sensitivity of the shadow response with its peak shifted toward shorter wavelength (Fig. 5); and (3) a more acute response to continuous changes in light intensity (Fig. 6). This last finding in particular suggests that small medusae do not use the slow decrease in light intensity at sunset to initiate upward movements since their predicted swimming frequency at these rates of change in light intensity is far too low. These findings, in addition to (1) the observed higher maximum swimming frequency during extended swimming bouts (Fig. 2); (2) greater maintenance swimming frequency (Arkett, 1984); and (3) a greater maximum velocity gain per swimming cycle (relative to their size) (Gladfelter, 1972), all suggest that smaller and presumably younger medusae are more truly planktonic and occupy shallower waters than older medusae. These characteristics should maintain newly budded and young stages of medusae in surface waters, thereby facilitating dispersal. The small number and size of the tentacles in these young medusae may limit them to capturing and eating smaller plankters. Indeed, large numbers of very small individuals are often observed in the surface waters near Bamfield Inlet. As medusae age, many of the juvenile photic traits appear to be altered, although the mechanisms by which these apparent ontogenic changes occur are unknown. Larger individuals do not show photokinetic differences (Fig. 4), have a narrower spectral sensitivity of the shadow response (Fig. 5), and show a less acute response to continuous changes in light intensity (Fig. 6). As already discussed, this last characteristic may explain the initiation of diel vertical migration of *P. penicillatus.* Furthermore, the lower swimming frequency during extended swimming bouts (Fig. 2), and lower "maintenance swimming" frequency (Arkett, 1984) suggests that they spend more time in deeper water. In shallow inlets, where *P. penicillatus* is commonly found, they assume a demersal existence, feeding on large demersal zooplankters (Arkett, 1984). Synchronized upward movement at night and concomitant spawning of these older reproductive individuals (Mills, 1983) may also ensure higher rates of fertilization.

In summary, *P. penicillatus* is most responsive to the rate of change in light intensity. In older medusae, the shadow response probably initiates diel vertical migration and does not function in predator avoidance. The function of the shadow response in young medusae remains unclear. That the shadow response of other hydromedusae (*e.g., G. vertens, S. atra, B. principis*) may function in the same way is suggested by their rapid upward movement during sunset (Mills, 1983). Additionally, the rapid downward movement of *P. penicillatus* and these other hydromedusae may be due to inhibition of swimming and to crumpling during sunrise. These movements are likely due to changes in the normal or "maintenance swimming" frequency alone.

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DNA SYNTHESIS AND THE ANNUAL SPERMATOGENIC CYCLE IN INDIVIDUALS OF THE SEA STAR *PATIRIA MINIATA*

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ABSTRACT

Male individuals of the sea star *Patiria miniata* have an annual spermatogenic cycle. This cycle was first suggested by gonad-index and histological analyses for a population of *P. miniata* in the field. The cycle was confirmed by tritiated-thymidine autoradiography of testes surgically removed about every 2 months from each of 10 individuals kept in the laboratory. Tritiated-thymidine autoradiography also revealed that the spermatogenic cycle includes a period of quiescence when sperm are not being produced and DNA synthesis is not occurring. Repeated surgical sampling of testes from individuals and autoradiography with tritiated thymidine are potentially useful methods for laboratory studies on the regulation of DNA synthesis and spermatogenesis and on regeneration.

INTRODUCTION

The study of spermatogenesis in asteroids can contribute significantly to our understanding of spermatogenesis in general and the factors regulating it. An annual spermatogenic cycle has already been established for many species of asteroids (*cf.* Shick *et al.,* 1981; Walker, 1982). Recently, several investigators have taken up the question of what exogenous or endogenous factors regulate spermatogenesis and the spermatogenic cycle in asteroids (Delavault and Buslé, 1970; Kanatani, 1973; Schoenmakers *et al.,* 1976; Kubota *et al.,* 1977; Walker, 1980; Pearse and Eernisse, 1982; Walker and Larochelle, 1984).

Patiria miniata, a gonochoric asterinid asteroid, can be a valuable animal for studies on spermatogenesis. In central and northern California it is abundant in protected intertidal rocky areas and subtidal kelp forests (Ricketts *et al.,* 1968; Pearse and Lowry, 1974; Gerard, 1976; Feder, 1980). It is extremely hardy and therefore suitable for laboratory studies, including those involving surgery and organ culture (*cf.* Davis, 1982). Previous studies indicate that *P. miniata* probably has an annual gametogenic cycle, although the cycles of different individuals are not very synchronous, and sperm and ripe eggs can be obtained from at least some animals at almost any time of the year (Farmanfarmaian *et al.,* 1958; Lawrence, 1965; MacGinitie and MacGinitie, 1968; Ricketts *et al.,* 1968; Nimitz, 1971, 1976; Gerard, 1976).

One of the questions addressed in the present study is whether DNA synthesis in germinal cells occurs throughout an annual spermatogenic cycle or is restricted to particular periods. These periods may be defined by the calendar or by other events in the cycle. Since the process of spermatogenesis involves many cell divisions and these are preceded by DNA synthesis, the starting and stopping of DNA synthesis in spermatogonia or spermatocytes may indicate the location and timing of factors regulating spermatogenesis. Autoradiography with tritiated thymidine, the technique used

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in this study, has previously been used for observations of DNA synthesis in testes at different points in the spermatogenic cycle in the sea urchin *Strongylocentrotus purpuratus* (Holland and Giese, 1965), the sea star *Asterias rubens* (Van der Plas *et al.*, 1983), and the brittle star *Amphipholis kochii* (Yamashita and Iwata, 1983).

In the present study DNA synthesis during the annual spermatogenic cycle was followed both for a population of animals in the field and for a group of individuals in the laboratory. The lab study was to determine whether the rather asynchronous individuals from the field population each have a cycle of spermatogenesis and DNA synthesis. Delavault (1963) repeatedly removed gonads surgically from individuals of the sea star *Asterina gibbosa.* It appeared that this technique would permit following the reproductive cycle in individuals of *Patiria miniata,* which has a body wall soft enough to be easily cut.

MATERIALS AND METHODS

Animal collection and maintenance

Individuals of *Patiria miniata* were collected subtidally by SCUBA from a kelp forest in the Hopkins Marine Life Refuge, at Point Cabrillo, on Monterey Bay, California. Animals with the longest arm between 60 and 80 mm long were collected approximately every two months for two and a half years (Fig. 1). These animals generally weighed between 50 and 120 g. Animals in this size range were used because in preliminary collections I found that in such animals there was no apparent trend of gonadal or pyloric cecal size changing with body size, while in smaller animals there were such trends (Davis, 1982). Sea stars that were not dissected at Hopkins Marine Station the day of collection were kept at Long Marine Laboratory of the University of California at Santa Cruz. In the laboratory the specimens were kept in tanks with fresh-flowing seawater in a room with fluorescent lights (G. E. F40D Daylight) set to come on at local sunrise and go off at local sunset. The temperatures of the seawater in the lab and in the field were indistinguishable. The animals were fed kelp, *Macrocystis pyrifera,* and smashed mussels, *Mytilus californianus.*

Dissection of field animals

For the study of the reproductive cycle in a population, at least 10 males from each of the collections were dissected within a week of collection (Fig. 1). After an animal was weighed and dissected, the following were recorded: wet weight of the 10 gonads; wet weight of the 10 pyloric ceca; and sex, determined from color of gonad (white in males or orange in females) and/or from the presence of sperm or oocytes in a squash. From these weights the gonad index (GI) and pyloric cecum index (PC-I) were calculated, each defined as the percent of the body weight comprised by the gonads or pyloric ceca, respectively. Data for females are presented in Davis (1982).

Testes from the males collected from February 1978 through February 1979 were fixed in seawater-Bouin's for histological analysis. Testes from males collected from April 1979 through July 1980 were incubated in tritiated thymidine for autoradiographic analysis (see below) (Fig. 1).

Surgical removal of testes from laboratory animals

For the study of the reproductive cycle in individuals, 20 Point Cabrillo males collected 18 April 1979 were prepared 23 April. *P. miniata* has 10 gonads, two per interradius. For an animal of unknown sex, I located in one interradius the medial

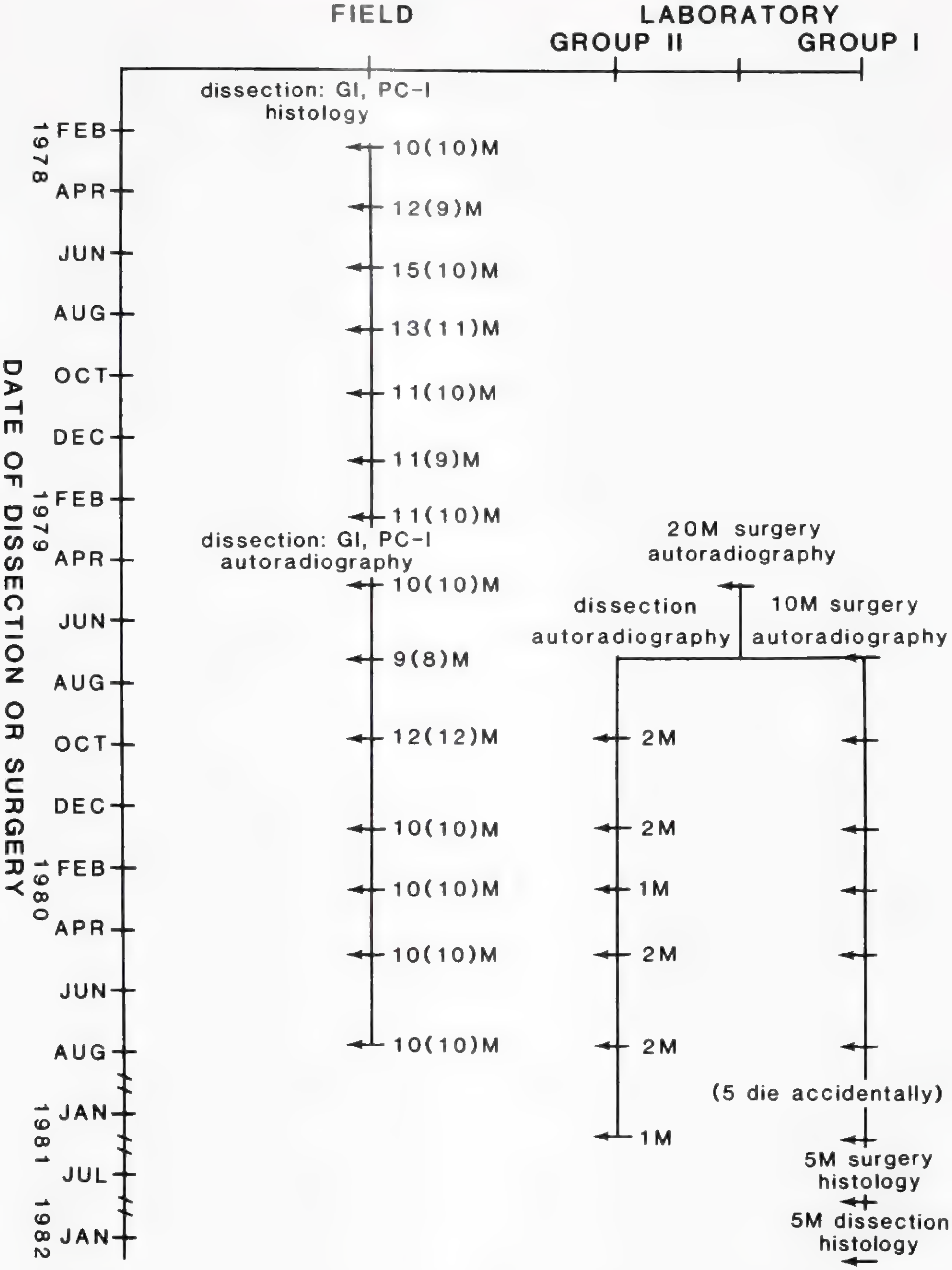
FIELD
LABORATORY
GROUP II
GROUP I
dissection: GI, PC-I
histology
1978
FEB
APR
JUN
AUG
OCT
DEC
1979
FEB
APR
JUN
AUG
OCT
DEC
1980
FEB
APR
JUN
AUG
1981
JAN
JUL
1982
JAN
DATE OF DISSECTION OR SURGERY
10(10)M
12(9)M
15(10)M
13(11)M
11(10)M
11(9)M
11(10)M
dissection: GI, PC-I
autoradiography
10(10)M
9(8)M
12(12)M
10(10)M
10(10)M
10(10)M
10(10)M
20M surgery
autoradiography
dissection
autoradiography
10M surgery
autoradiography
2M
2M
1M
2M
2M
1M
(5 die accidentally)
5M surgery
histology
5M dissection
histology

FIGURE 1. Design of the studies of the reproductive cycle in *Patiria miniata.* Animals were either collected from the field approximately every two months, or were collected in April 1979 and then kept in the laboratory. Included are the dates that gonads were removed from animals by surgery or dissection; the numbers of animals used; and the methods of analyses of the gonads, *e.g.*, gonad and pyloric cecum indices, histology, autoradiography. When two numbers are shown for the number of males, the first is the number used for the organ indices, the second (in parentheses) is the number also used for histologic or autoradiographic analyses. M = male. GI = gonad index. PC-I = pyloric cecum index.

line of ossicles that indicates the underlying internal pillar and cut a wedge-shaped flap, the apex pointing outwards, about 1 mm to either side of the medial line. The apex was lifted with forceps, while the gonoduct, attached to the underside of the flap, was gently pulled from the animal along with the duct's gonad. The gonad was then sexed; and for each of the first 20 males, the removed testis was incubated in tritiated thymidine, the wedge-shaped flap was folded back into place, and one or more notches was cut into the side of one or more rays to identify the individual. The 20 males were kept together in one tank.

In July 1979 the sea stars in the common tank were divided into 2 groups of 10 males each, the groups equivalent in their animals' sizes and reproductive states. The two groups were designed to examine for any effects of the frequency of surgery on reproductive state. From each of the 10 sea stars in Group I, a testis was surgically removed and analyzed autoradiographically about every two months through July 1980 at about the same time as the sampling of testes from the field animals (Fig. 1). The first five times testes were removed surgically from a particular individual, the testes were taken from a different interradius each time. Five animals died in the fall of 1980 when the air to the tank accidentally went off. From each of the five remaining individuals, testes were removed surgically in January and July 1981; and the animals themselves were dissected in January 1982 (Fig. 1). The 10 males in Group II were not surgically sampled again after 23 April 1979 but were dissected at various times: one or two animals about every two months through July 1980, the tenth animal in January 1981 (Fig. 1). For both Groups I and II, whenever a testis was surgically removed from an interradius from which a testis had been taken at an earlier date, or any time an animal was dissected, any regeneration of previously removed testes was noted.

Histology and autoradiography

Usually one entire testis per animal was used for the incubations in tritiated thymidine. In some sampling periods, however, two or three testes from each animal were used. Comparisons among the multiple testes within an animal, including comparisons between intact testes and testes in pieces, showed no obvious difference in distribution of radioactive label (Davis, 1982).

Each testis was incubated two hours in 5–15 ml of 3 μC/ml of tritiated thymidine in seawater. The incubation flasks were shaken in a water bath cooled by cooling coils hooked up directly to a spigot of the laboratory's seawater system. The temperatures of the seawater in the water bath for incubations from April 1979 through January 1981 ranged from 12.9 to 15.9°C and were always within 0.5°C of the temperature of the seawater in the laboratory's seawater system. After incubation, the tissues were fixed in seawater-Bouin's.

Following fixation, the tissues were dehydrated in an alcohol series, embedded in Paraplast Plus, and sectioned at 7 or 10 μm for histological analysis or 5 μm for autoradiographic analysis. The autoradiographic slides were then dipped in Kodak NTB-2 emulsion and exposed to the emulsion for two to eight weeks. All slides were stained with standard alum hematoxylin (Galigher and Kozloff, 1971) and eosin.

Analysis

To minimize bias in my descriptions of reproductive states, I mixed together the slides from all samples and covered their labels. The labels were uncovered only after all slides were described. While there was some variation in reproductive state between lobes within a testis, it was not difficult in most cases to assign a reproductive state on the basis of a clear majority of lobes.

Each testis was ranked according to stage in the reproductive cycle. Several criteria were used in this description. These included the following histological characteristics of the germinal epithelium: presence or absence of spermatogenic columns, the types of germinal cells within the columns, the thickness of the spermatogonia-spermatocyte layer relative to the thickness of the spermatid layer, and the overall thickness of the germinal epithelium. The abundance of sperm in the lumen was also noted. Additional criteria were the proportion of spermatogonia and spermatocytes labeled with tritiated thymidine, and the thickness of the labeled cell layer relative to the thickness of the unlabeled cell layer.

On the basis of the above parameters, I rated the reproductive state of each testis with a number from 0.0 to 3.5, where 0 = inactive, 1 = early spermatogenesis, 2 = active spermatogenesis, 3 = ripe. The distinguishing characteristics of the categories are shown in Table I. Testes intermediate between two of these states or with mixtures of lobes differing in state by as much as one whole number were given intermediate numbers. As a 0.0 (inactive) was considered equivalent to a hypothetical 4.0 (spent), testes intermediate between 3.0 and 0.0—that is, partially spawned ones—were rated 3.5.

RESULTS

Reproductive cycle in the Point Cabrillo population

There was considerable individual variability among the gonad and pyloric cecum indices of male *P. miniata* collected over a period of two and a half years (Fig. 2). The mean gonad index differed significantly from month to month (ANOVAs, $P < .01$), but the month in which it was highest and the highest value reached varied from year to year. The eight highest mean gonad indices occurred from December through June, the six lowest from July through December. Thus, Figure 2 suggests that spermatogenesis occurred from early or late fall to early spring, and spawning in late spring or early summer. The mean pyloric cecum index differed significantly from month to month (ANOVAs, $P < .01$), but there were no consistent year-to-year trends (Fig. 2).

Testes of various reproductive states from *P. miniata* are shown in Figure 3A–F. The histology is similar to that in other asteroids (*e.g.*, Pearse, 1965; Walker, 1980).

TABLE I

Characters used to distinguish reproductive states of testes from Patiria miniata

Reprod. state	Columns	Types of cells in g.e.	Proportion of G and C labeled	Relative thicknesses of cell layers	Abundance of sperm in lumen
0.0	No	G	few	– –	generally few
1.0	No	G	many	– –	– –
1.5	Yes	G, C	most	– –	– –
2.0	Yes	G, C, T	most	T < 1/2 g.e.	– –
2.5	Yes	G, C, T	most	T > 1/2 g.e.	– –
3.0	No	G, C, or T	variable	– –	many

G = spermatogonia. C = spermatocytes. T = spermatids. g.e. = germinal epithelium. The "– –" in columns denotes characteristics that were not used to distinguish states.

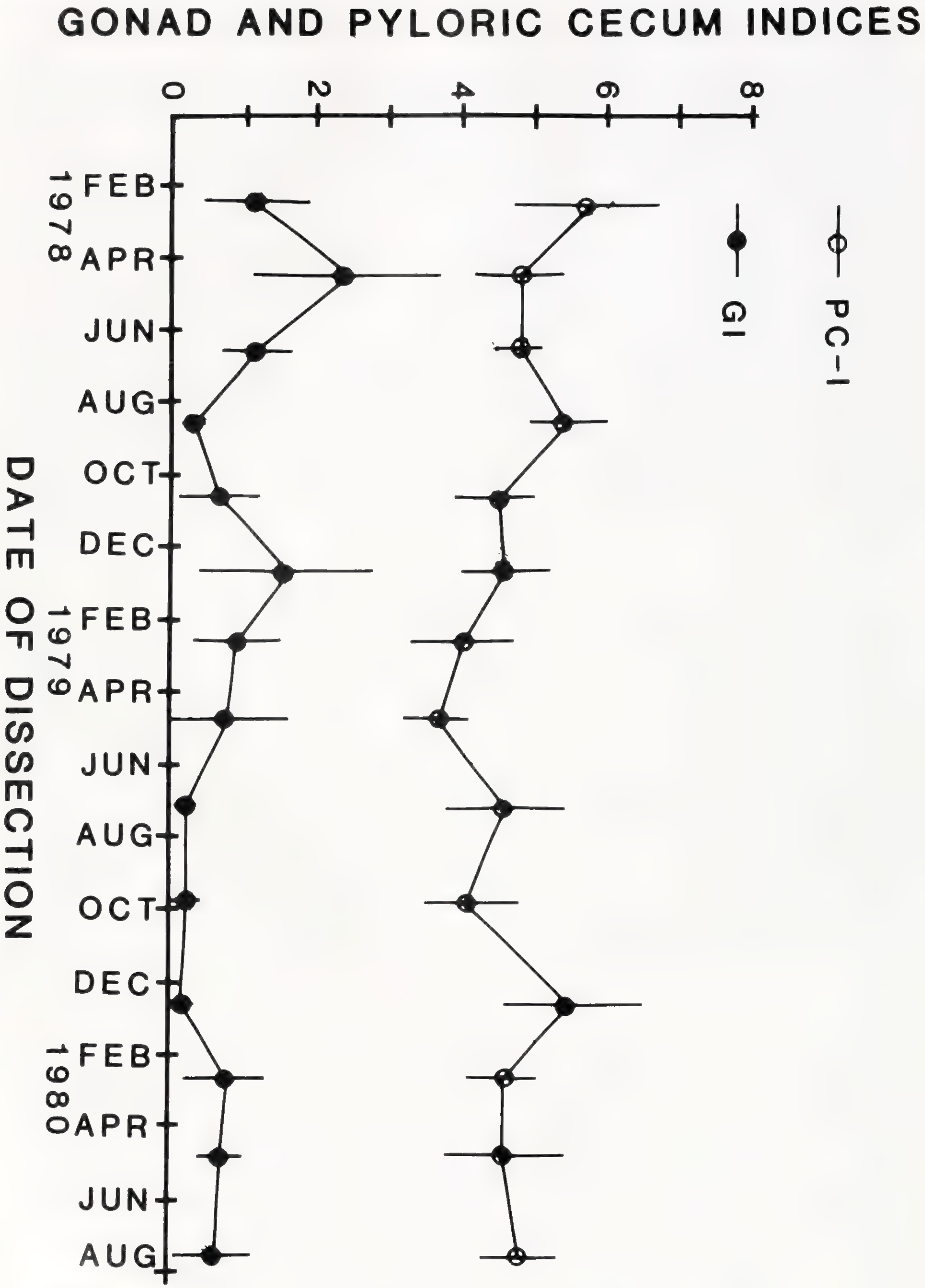

FIGURE 2. Gonad and pyloric cecum indices of *Patiria miniata* collected from the field approximately every two months. Mean ± 95% confidence limits. GI = gonad index. PC-I = pyloric cecum index.

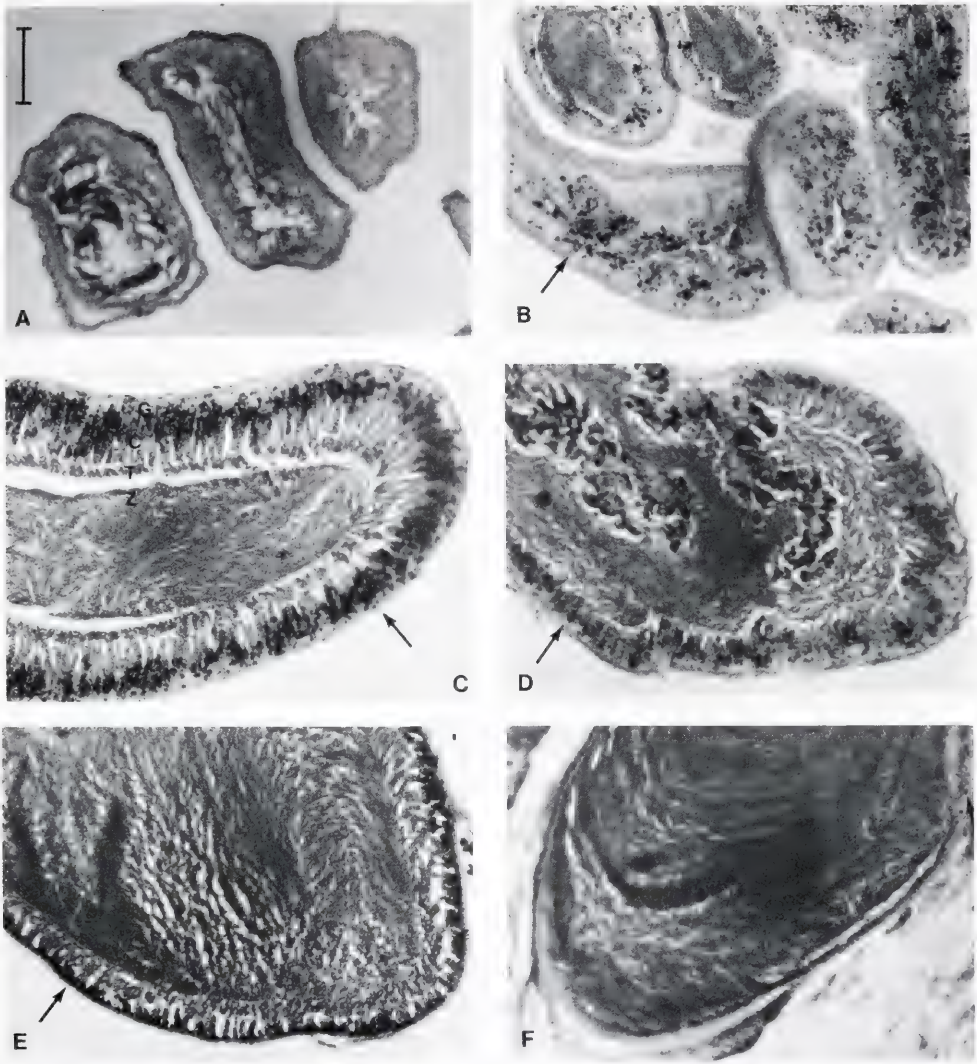

FIGURE 3. Autoradiograms showing various reproductive states of testes from *Patiria miniata.* Arrows indicate areas with silver grains representing DNA synthesis. Scale = 100 μm. A: 0.0 (inactive). B: 1.0 (early spermatogenesis). C: 2.0 (active spermatogenesis). G, C, and T indicate areas of the spermatogonia, spermatocytes, and spermatids, respectively, in the spermatogenic columns of the germinal epithelium. Z indicates sperm in the lumen. D: 2.0 (active spermatogenesis), with branching germinal epithelium. E: 2.5. F: 3.0 (ripe).

A testis consists of lobulated sacs. Around the inner wall of a testicular lobe is the germinal epithelium, containing various types of germinal cells, which may be distinguished from one another on the basis of position, size, appearance of chromatin, and staining intensity. In a testis actively producing sperm (*cf.* Fig. 3C), the germinal epithelium is comprised of columns, with the spermatogonia at the base, followed distally by spermatocytes, then spermatids. In a 2.0 testis, most of the spermatocytes closer to the bases of the columns have radioactive label over them, while fewer of the spermatocytes closer to the spermatids do (Fig. 3C). Sperm are in the lumen of the testis. Sectioned germinal epithelium in 2.0 testes was frequently seen in the lumen. When I traced it in serial sections of some testes, I found that it joined the germinal epithelium around the inner wall of the testis. The germinal epithelium in the lumen resulted in some cases from the infolding of the hemal space, which surrounds the germinal epithelium in the wall; in other cases it arose from the branching of spermatogenic columns (Fig. 3D).

The reproductive states of the animals sampled from the field approximately every two months from February 1978 through July 1980 are shown in Figure 4. Prior to April 1979 the ratings were based on histology; after April 1979 they were based on both histology and autoradiography. The main consequence of this switch of criteria is that some of the testes rated 0.0 (*e.g.*, in August and October 1978) probably had some DNA synthesis and therefore would have been rated 0.5 to 1.0 with autoradiography. States 1.5 to 2.5 were the most spermatogenically active testes. That is, these had spermatogenic columns and more cells synthesizing DNA than the other states. In testes of states beyond 2.5, the germinal epithelium was nearing the end of the spermatogenic cycle, while in those below 1.5 it was either inactive (0.0) or beginning synthetic activity (1.0) (Fig. 3, Table I).

In general, stage 0.0 predominated in summer, 1.0 in late fall, 2.0 in winter, 2.5 in spring, and 3.0 in late spring. However, the timing of these stages varied from year to year. For example, testes in December 1978 were more advanced than those in December 1979, and testes in April 1979 and 1980 were more advanced than those in April 1978. There was also individual variability; not all animals in a sample were at the same stage of reproductive development (Fig. 4).

Gonad index was not a good indicator of reproductive state except that gonad indices exceeding 0.5 almost always indicated testes of states 2.0–3.0 (Fig. 5). In general, there was no relationship between pyloric cecum index and reproductive state. However, while the mean pyloric cecum index for reproductive state 0.0 was similar to the mean indices for individuals in more developed reproductive states, the lowest indices—three or less—all were associated with state 0.0 (Fig. 5).

Reproductive cycle in the laboratory animals

An annual progression of spermatogenic stages was also observed in the *Patiria* kept in the laboratory (Fig. 4). Furthermore, the cycle could be followed within individuals. Thus, of the 10 sea stars in Group I, 6 were spermatogenically active in September and December 1979 and in February and April 1980; 2 (individuals #9 and #17) lagged behind; 1 (#19) may never have produced sperm during the year; and 1 (#10) clearly did not (Fig. 6). At each sampling period from September 1979 through January 1981, the reproductive states in Group II (the laboratory animals sampled surgically in April 1979 and not again until dissected) were comparable to those in Group I (Fig. 6, Table II). However, the testes of both groups of laboratory animals were clearly more advanced than those in the field animals in September and December 1979, even though there was little difference in July 1979 (Figs. 4, 6; Table II).

From July 1980 through January 1982, four of the five Group I animals that were sampled every six months continued spermatogenesis until the tenth testis was removed from the body, while the fifth animal, individual #17, apparently did not (Fig. 6). These four animals had testes of reproductive states 1.5–2.0 in January 1981 and January 1982, which was in accord with the states of testes taken at that time of year from the lab animals in the previous year and from the field animals in the previous two years (Figs. 4, 6). However, in July 1981, testes from three of the four animals had testes of reproductive states 2.0–2.5 (Fig. 6). This was surprising since the field

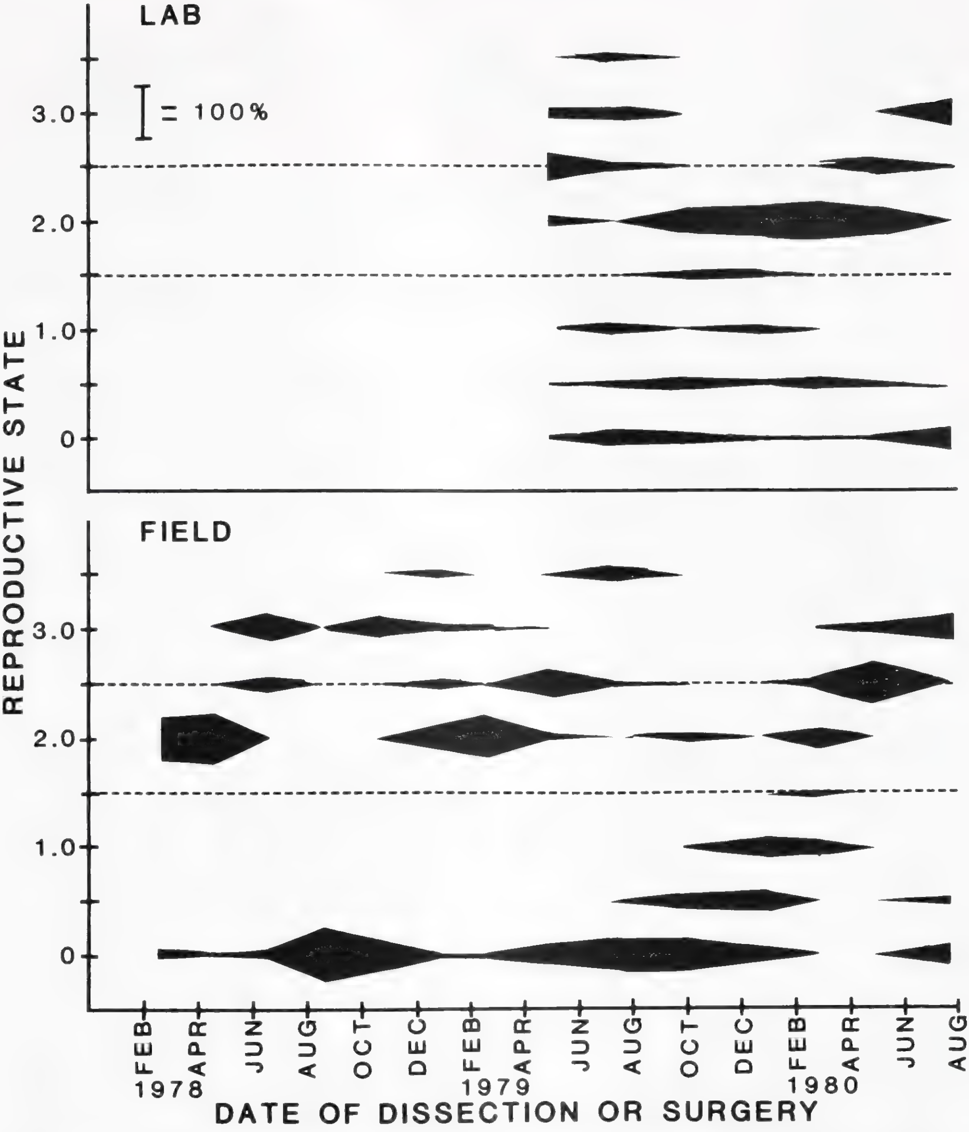

FIGURE 4. Reproductive states of testes from *Patiria miniata.* The testes were taken by dissection from field animals or by surgery from Group I laboratory animals. The width of the polygons represents the percent of the individuals in a sample that had testes of the reproductive states indicated. The scale indicates the width corresponding to 100%. Reproductive states from 1.5 through 2.5, indicated by the area between the dashed lines, represent the most spermatogenically active testes.

and laboratory data from the previous three summers had indicated that spermatogenic columns were usually absent from testes in July (Fig. 4).

Regeneration of gonads

The data on regeneration (or lack thereof) for the testes surgically removed from the lab animals were inconsistent. When the 10 Group II animals were dissected at

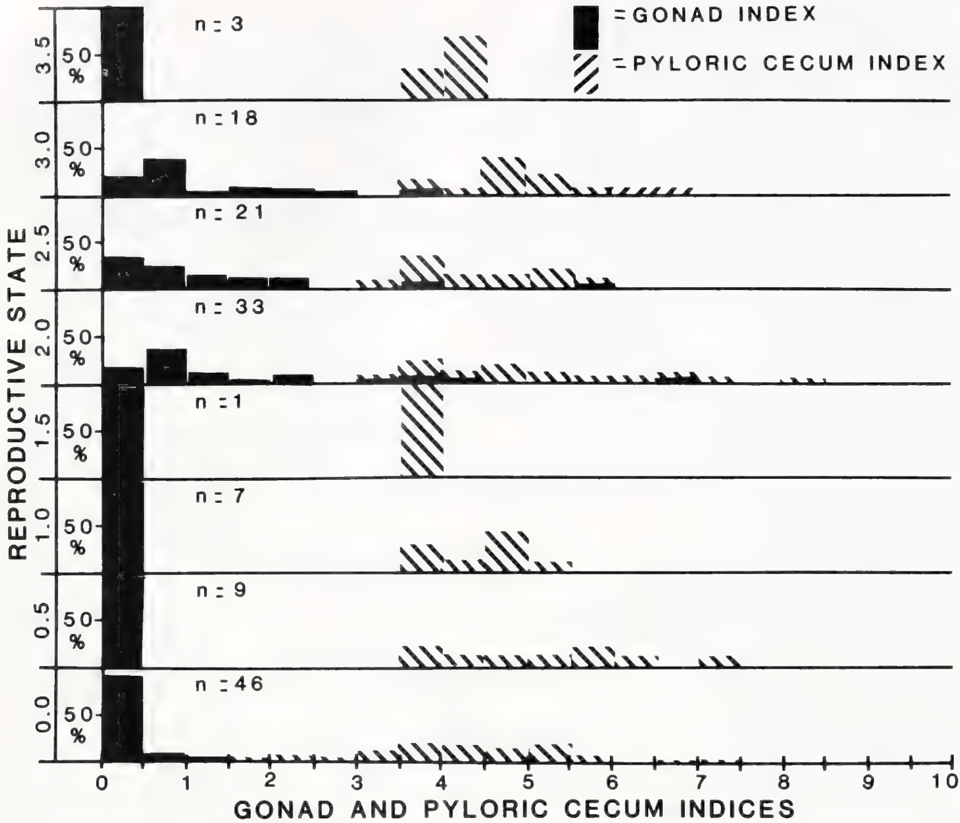

FIGURE 5. Gonad and pyloric cecum indices *versus* reproductive state for male *Patiria miniata* collected from the field between February 1978 and July 1980. The height of a column represents the percent of the individuals with the reproductive state indicated that had organ indices in the increments indicated.

various times between September 1979 and January 1981, only twice, once each in April and July 1980, was some testis found at the position from which a testis had been removed in April 1979. In Group I animals, when the second of the two testes at an interradius was surgically removed, there was, in 23 of 25 observations, no testis at the position from which the other testis at that interradius had been removed a year or more earlier. When the five Group I animals were dissected in January 1982, three of the animals clearly had some testis at one to six positions from which testes had previously been removed; but the number of presumably regenerated testes did not appear related to the time since removal of the testes.

Effects of surgery

Surgery appeared to have little adverse effect upon the behavior of the sea stars. Immediately after surgery, the animals were put on their backs in the tanks to see whether they could right themselves normally. They did so readily. They also crawled up the sides of the tank as usual. Smashed mussels were not added to the tank the week or two after surgery; but when they were, they were quickly eaten.

How fast the wounds healed depended upon how many of the edges of the wedge-

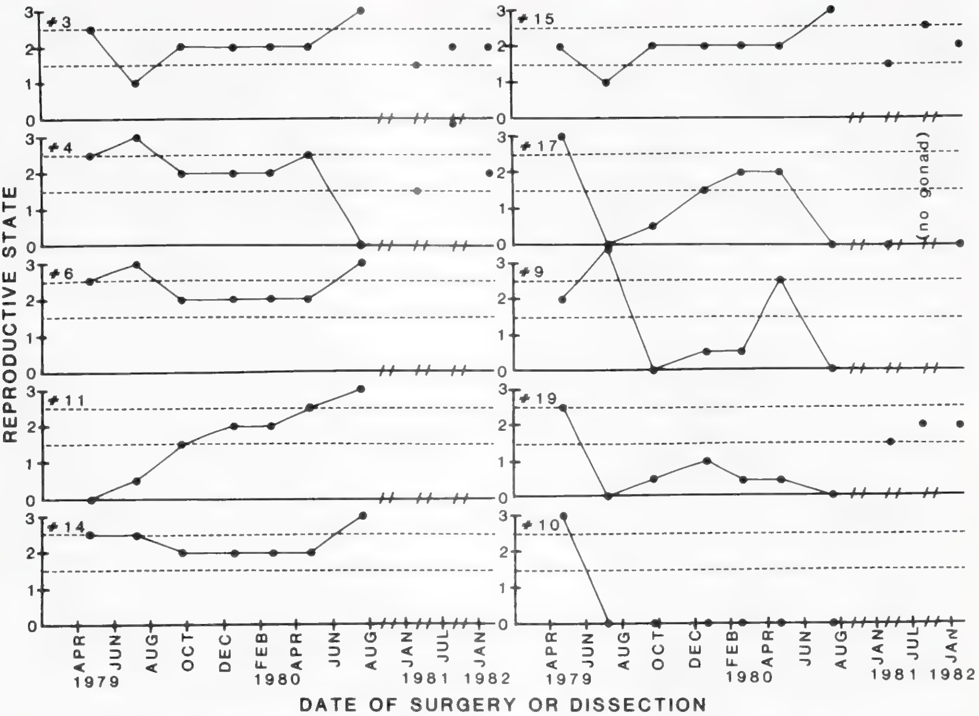

FIGURE 6. Reproductive states of testes from Group I individuals of *Patiria miniata* kept in the laboratory. Each animal was surgically sampled approximately every two months from April 1979 through July 1980, then every six months through dissection in January 1982. Each graph represents the testes from an individual whose identifying number follows the "#" symbol. (Five animals died accidentally in the fall of 1980.) Reproductive states from 1.5 through 2.5, indicated by the area between the dashed lines, represent the most spermatogenically active testes.

shaped flap contacted the edges of body wall surrounding the hole. If all edges of the flap made contact, the wound was often completely sealed within a week and sometimes difficult to see in two to three weeks. After the first surgical sampling in April 1979,

TABLE II

Reproductive states of testes from Group II Patiria miniata*—laboratory-maintained animals, each sampled surgically in April 1979, then sampled again when dissected*

Animal number	Reproductive state in April 1979	Month and year dissected	Reprod. state when dissected
1	2.5	Sep 79	2.0
5	2.5	Sep 79	2.0
2	0.0	Dec 79	2.0
7	2.5	Dec 79	2.0
12	2.5	Feb 80	2.0
8	2.5	Apr 80	2.0
13	2.5	Apr 80	2.5
16	0.0	Jul 80	1.0
18	3.0	Jul 80	3.0
20	2.5	Jan 81	2.0

no pyloric cecum hung out the surgical holes. However, later in the experiment, some pyloric cecum often would hang out after surgery, apparently because the ceca were larger then than at the beginning of the experiment. As a result, the wall flap was less likely to fit completely into place. When this post-surgical misfit occurred, complete sealing took longer; but the seal was always complete before the next sampling two months later.

Occasionally, the edges of the wedge-shaped patch of body wall became slightly necrotic, as evidenced by white puffiness, and in a very few cases this necrosis spread to areas of the body wall surrounding the flap. The development of inflammation in sea stars in response to injury has been discussed by Bang (1982).

DISCUSSION

Spermatogenic cycle in individual Patiria miniata

The results of the present study demonstrate an annual spermatogenic cycle in individual *P. miniata.* This cycle was first suggested by gonad-index and histological analyses for a population of *P. miniata* in the field. The cycle was confirmed by autoradiographic analyses of testes surgically removed from individual animals kept in the laboratory.

Mean gonad indices determined approximately every two months for male *P. miniata* collected from the Point Cabrillo kelp forest suggested that spermatogenesis occurs primarily in winter, and spawning in late spring and early summer. Individuals are quite asynchronous. These conclusions from the present study are in agreement with those of previous workers on *P. miniata,* including the two-year study by Gerard (1976) in the same subtidal study area as the present one; the two-year study in an intertidal area in Tomales Bay by Nimitz (1971); and the one-year studies in intertidal areas near Point Cabrillo by Farmanfarmaian *et al.* (1958) and Lawrence (1965).

My histological studies of the testes from *Patiria miniata* corroborated the impression from the measurements of gonad index that spermatogenesis occurs from fall through spring. The seasonal spermatogenic cycle was evident both in testes taken every two months from animals collected from the field, and in testes of animals kept in the laboratory. Nimitz (1976) reported similar results for the cycle in *P. miniata* in the field. In the present study, good criteria for characterizing the reproductive state of a testis were the presence or absence of spermatogenic columns and the proportions of cell types present in the columns. Measurements of the thickness of the germinal epithelium in *P. miniata* were not useful because of the variability within a testis and often within a lobe. Shick *et al.* (1981) reached the same conclusions about the thickness of the germinal epithelium in testes of *Ctenodiscus crispatus.*

The annual progression of reproductive stages determined from the sampling of testes from seasonal collections of *P. miniata* from the field was confirmed by the autoradiographic analyses of testes surgically sampled from individuals kept in the laboratory. The timing of the onset of spermatogenesis in laboratory animals varied somewhat with the individual, and one or two of the animals did not produce sperm at all (*cf.* Fig. 6). Unfortunately, these two animals died in a water-supply accident in the fall of 1980, so it was not possible to determine whether their gonads would have developed the following year. However, another animal kept in the laboratory as part of a different study did not undergo gametogenesis its first year in the lab even though it appeared to be sexually mature, but did the second year. That animals may sometimes skip a gametogenic cycle suggests that occasional apparently sexually mature winter animals with spermatogenically inactive testes (*cf.* Fig. 4) may be skipping the spermatogenic cycle that year, rather than spawning several months out of synchrony with the rest of the population.

Most of the laboratory animals began spermatogenesis more than two months before those in the field. The reasons for this are not clear but may include any of the following: (1) the animals in the laboratory may have spawned earlier than those in the field, and consequently began the next cycle earlier. (2) The surgical removal of testes from the laboratory animals in April and July 1979 may have caused the remaining testes to develop precociously by September and December 1979, whether due to compensation for missing gonads or release from some inhibition. (3) The environment of the laboratory animals may have differed from that of the field animals in such factors as temperature or the quality or quantity of food.

DNA synthesis during the spermatogenic cycle

The results of the present study also show that germinal cells appear to have a clearly delineated quiescent period in which no sperm are produced and no DNA synthesis occurs. Testes without spermatogenic columns could be divided into two states based upon the absence or presence of DNA synthesis in the germinal epithelium (states 0.0 and 1.0, respectively). Walker (1980) reported that somatic cells with $\Delta 5\text{-}3\beta$-hydroxysteroid dehydrogenase activity appeared in the testicular lumen of *Asterias vulgaris* just before spermatogenic columns formed. Perhaps these somatic cells are associated with the initiation of DNA synthesis in germinal cells.

DNA synthesis was always occurring in all of the spermatogenic columns in the 2.0 testis in *P. miniata.* Radioactive label, indicating DNA synthesis, was generally seen over most spermatogonia, most spermatocytes near the bases of the columns, and fewer but many spermatocytes near the tips of the columns. This distribution of label has also been seen in testes of *Asterias vulgaris* (Walker, 1980), *A. rubens* (Van der Plas *et al.*, 1983), and *Leptasterias pusilla* (Smith, 1971). Walker (1980) found that germinal cells move from the bases toward the tips of the columns during spermatogenesis. Therefore, unlabeled spermatocytes nearer the spermatids are presumably past DNA synthesis.

In the germinal epithelium of stage 2.5 testes in the present study, the thickness of the spermatid layer was greater than that of the spermatogonia-spermatocyte layer. This suggests that, as described by Walker (1980) for *A. vulgaris,* mitoses in the spermatogonia were occurring at a slower rate than were meioses in the spermatocytes. Nevertheless, in *P. miniata* DNA synthesis continued in both the spermatogonia and spermatocytes as the columns were degrading in 2.5 testes. DNA synthesis also occurred in some cells in the germinal epithelium in 3.0 testes, after the columns were gone. Whether DNA synthesis was followed by cell division is not known.

Regeneration of testes

In the present study a variable degree of removal of the genital system along with the gonoduct and testis was probably responsible for the observed variable regeneration of the gonad. Hauenschild (1954) found that the gonads removed from the asterinid *Asterina gibbosa* did not regenerate, while Lender and Huet (1962a, b) and Huet (1965) reported that they did. Okada (1979) found for the sea urchin *Hemicentrotus pulcherrimus* that the degree of regeneration of a removed gonad depended upon the extent of the removal of the gonoduct and blood vessel [= hemal system] to the gonad.

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THE MATING SEQUENCE OF THE BENTHIC ARROWWORM *SPADELLA SCHIZOPTERA*

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ABSTRACT

The mating behavior of the hermaphroditic arrowworm, *Spadella schizoptera,* was followed photographically. The mating behavior consists of four distinct actions: vertical swinging of the worm's body, touching each other with their heads, standing erect face-to-face, and sperm transfer. During sperm transfer, one worm (donor) jumps suddenly, ejaculates a sperm cluster, and transfers it to the partner (recipient). The well-ordered mating sequence leads to accurate placement and transfer of the sperm to the genital orifice of the partner. We concluded that sperm transfer in *Spadella schizoptera* is non-reciprocal: one worm acts as a male and the other as a female.

INTRODUCTION

The chaetognaths, or arrowworms, are mostly planktonic and, in general, are not easily maintained in the laboratory. Behavioral aspects of their reproduction such as mating and egg-laying have been relatively ignored, compared to our understanding of the maturation process of sperm and eggs (Stevens, 1910; Burfield, 1927; John, 1933; Jägersten, 1940; Ghirardelli, 1968; Alvariño, 1983a, b).

The arrowworms are hermaphroditic; some species are capable of both cross- and self-fertilizations (*cf.* Reeve and Cosper, 1975). For the planktonic species, *Sagitta hispida,* Reeve and Walter (1972) have shown photographs of worms just before and after sperm transfer. The mating behavior of the benthic species, *Spadella cephaloptera,* has been studied by Vasiljev (1925), John (1933), and Ghirardelli (1953, 1968). According to Vasiljev (1925) and Ghirardelli (1953, 1968), sperm transfer is reciprocal, each worm acts simultaneously as a male as well as a female. On the other hand, John (1933) has described it as non-reciprocal: one worm acts only as a male and the other, only as a female.

While breeding *Spadella schizoptera* in the laboratory, we found that mating behavior is different from that reported for *S. cephaloptera.* We describe an ordered sequence of mating that leads to sperm transfer in *S. schizoptera.*

MATERIALS AND METHODS

Specimens of *Spadella schizoptera* were collected in March of 1983 and 1984 from tide pools near the Amakusa Marine Biological Laboratory, Kumamoto Pref. The temperature of the seawater was about 14°C. All animals were taken to the Ushimado Marine Laboratory and kept in seawater-filled vessels (25 cm diameter, 10 cm depth) in a room maintained at a constant temperature (14°C) having a cycle of 12 h light and 12 h darkness. Ten worms were placed in each vessel. Seawater was changed every three days, at which time food (either *Artemia* nauplii or *Tigriopus*) was added. Only mature worms were used for behavioral observations. In these worms, the seminal

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vesicles were completely filled with sperm, although the ovaries contained varying sized ova.

Mating behavior was induced when more than three worms in 50 ml seawater were transferred to room temperature (approximately 18–20°C). Successful sperm transfer was observed more than a hundred times. In order to follow the sequence of mating behavior, 10 mature worms were placed in a square vessel (8 cm × 8 cm, 3 cm depth) and photographed from the side at 2–5 s intervals. The camera system enabled single as well as multi-exposures using strobe flashes.

Transferred sperm clusters were observed using a Hitachi 610 scanning electron microscope. For this purpose, we fixed worms which had not mated for several hours, worms which had just received a sperm cluster, and worms which were laying eggs. All specimens were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer containing 0.4 *M* sucrose (pH 7.4) and rinsed in the same buffer for 2 h each. After dehydration through a graded series of ethanol followed by substitution with isoamyl acetate, the worms were dried in a critical point drying apparatus and coated with gold.

RESULTS

Unless disturbed, *Spadella schizoptera* stays on a substrate, usually attached by its adhesive fins. Sometimes they detach and dart a short distance. When the worm population density and the seawater temperature were raised, the worms became active swimmers, reattaching to the substrate occasionally. In a resting state, some of those worms began to swing their bodies vertically 2–5 times, while remaining attached to the substrate using the adhesive fins as a fulcrum (Fig. 1A, right worm). In general, these movements lasted approximately 1–2 s, and recurred several times with only a few seconds interruption. When two worms came within a distance of about 5 mm, they often started a mating sequence as shown in Figure 1a–f. In such cases, at least one of the worms exhibited the swinging movement. The second worm, which had not swung its body previously, started a swinging movement in response. The initial swinging movements thus appear to be a kind of signaling behavior. The two worms then moved nearer to each other (Fig. 1b).

The two worms eventually contacted one another with their heads (Fig. 1c), then moved closer and suddenly stood erect, assuming a face-to-face (ventral to ventral) position (Fig. 1d). After about 5 to 30 s, one sprang up momentarily and hit its partner with its tail (Fig. 1e). Immediately after the hit, a sperm cluster appeared on the side of the worm that was hit (Fig. 1f, the arrow on the left worm), while the other worm exhibited seminal vesicles which appeared largely exhausted as evidenced by changes in color and size. The hitting action appears to transfer the sperm; we will refer to the worm that hits the partner as a donor and the other one as a recipient. After sperm transfer, the two worms swam apart. The whole sequence of mating behavior varied in duration, from less than 10 s to a few min. On rare occasions, the same couple would immediately remate by reversing their roles as donor and recipient.

The transfer of a sperm cluster is the most important event in the mating sequence. The worm has a pair of seminal vesicles and it is possible for the donor to transfer two clusters to the recipient during a single mating. Figure 2 shows a successful case of a simultaneous double transfer. The transfer process was too rapid to be photographed. Sperm were released as a cluster from the apical region of the seminal vesicle in response to touching the seminal vesicle gently with a needle (Fig. 3a, b). Sperm cluster attachment always occurred on the ventral side of the body, just anterior to the lateral fins (see Fig. 2). SEM observations of egg-laying worms revealed a large pore (70 μm diameter, Fig. 4) which corresponds to the region where the sperm cluster

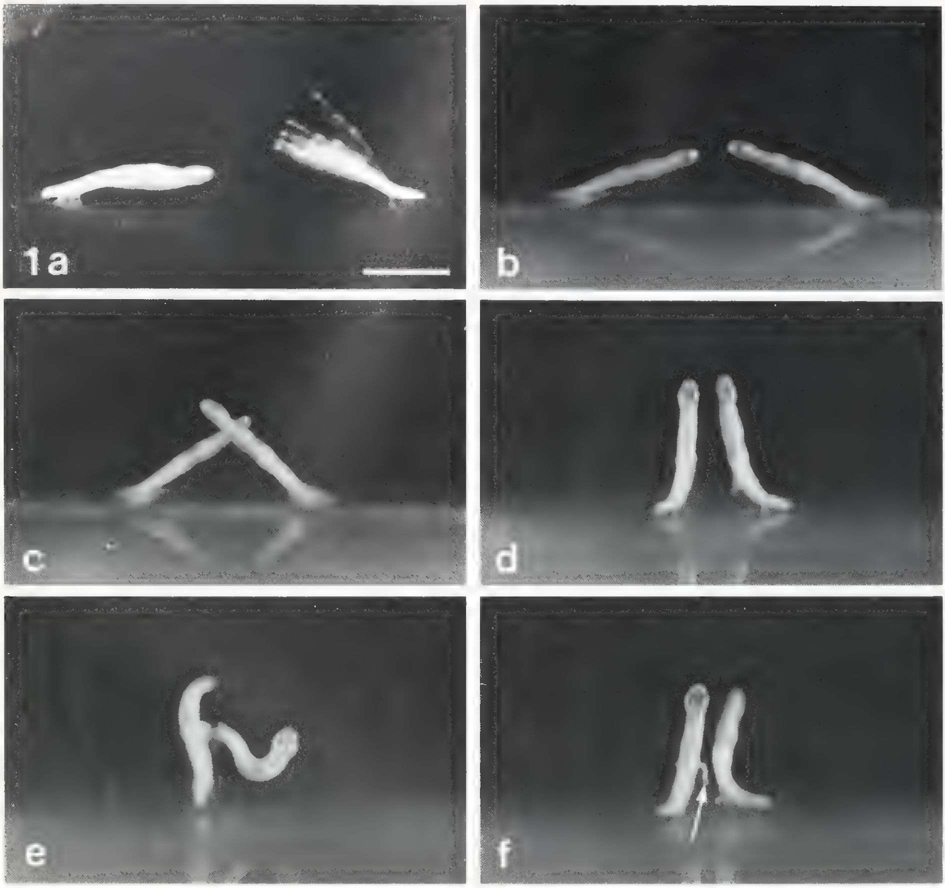
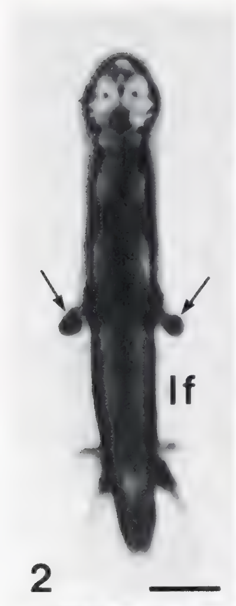


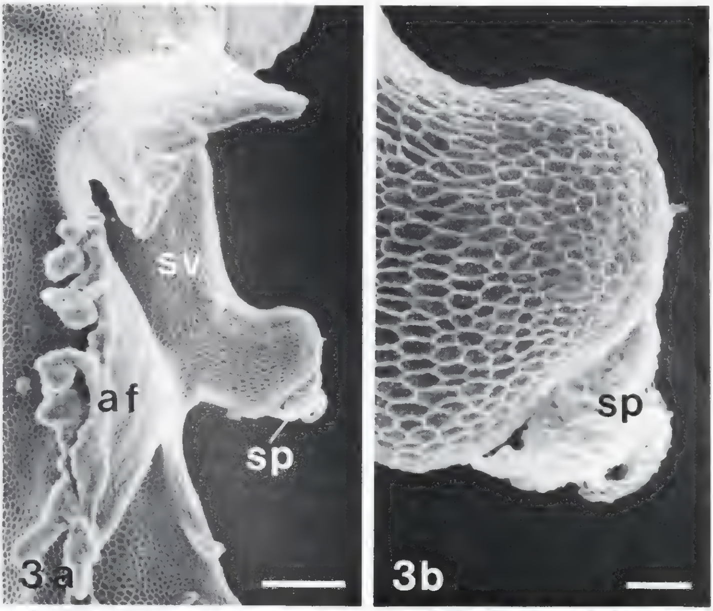
FIGURE 1. Sequence of mating behavior of *Spadella schizoptera.* (a) Two worms approach, the right worm has initiated swinging movements. (b) The two worms move nearer to each other. (c) They eventually touch each other with their heads. (d) Suddenly they become erect, assuming a face-to-face position. (e) The right worm hits its partner with its tail. (f) The left worm can be seen with a sperm cluster (arrow) on its side. The time required from encounter (a) to copulation (e) was from less than 10 s to a few min. The pictures, (a), (b, c), and (d, e, f) were taken with different couples. Scale bar: 2 mm.

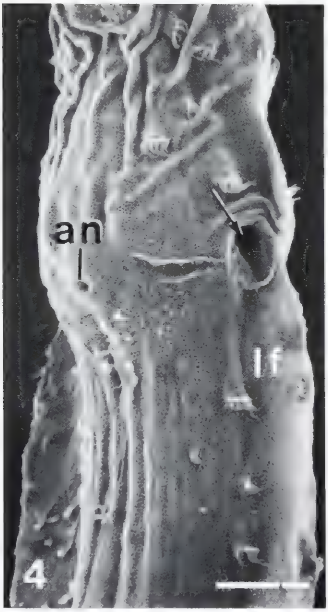
was to be attached (arrows in Fig. 5a). The pore appears to be enlarged at the time of egg-laying. The observed pore is apparently a genital orifice through which sperm enter (Fig. 5b) and eggs exit.

DISCUSSION

Spadella schizoptera can be maintained in the laboratory for several months. Mating behavior is frequently observed when the worms are placed in conditions of high density and elevated temperature. According to Ghirardelli (1968) the mating behavior of *S. cephaloptera* occurs mostly in the late afternoon. In its natural environment, *S. schizoptera* is found in small populations of about 10 to 20 worms per 900 cm^2 in the lower intertidal zone. Such population densities are not high, but apparently are adequate for conspecific contacts. Moreover, the temperature of tide pools rises during







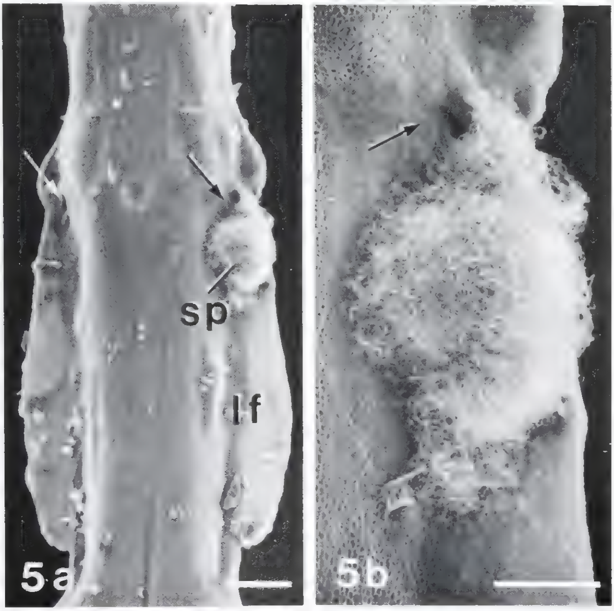


FIGURE 2. Dorsal view of a worm that received a pair of sperm clusters (arrows) which were attached to the ventral side, a little anterior to the lateral fins (lf). Scale bar: 500 μm.

FIGURE 3. (a) SEM of the seminal vesicle (sv) observed from the ventral side. At higher magnification (b), the sperm are just extruded from the apical region of a seminal vesicle which was touched with a needle. af, adhesive fin; sp, sperm. Scale bars: a = 50 μm; b = 10 μm.

FIGURE 4. SEM of the ventro-lateral region, a little anterior to the lateral fin of an egg-laying worm. The genital orifice (arrow) is clearly seen. an, anus; lf, lateral fin. Scale bar: 100 μm.

FIGURE 5. (a) SEM of the ventral region of a worm which has just received sperm (sp). The sperm which were pushed out as a cluster are attached near one of the genital orifices (arrows). (b) Higher magnification of the genital orifice (arrow) through which sperm appear to be entering. lf, lateral fin. Scale bars: a = 100 μm; b = 50 μm.

the day. This daytime condition resembles the conditions which induce mating behavior in the laboratory.

During mating of *S. schizoptera*, four actions occur sequentially: vertical body swinging, head touching, body alignment, and sperm transfer. The swinging movements (Fig. 1a, right worm) are repeated several times until sperm transfer is induced. These movements may be a key stimulus for the initiation of the mating sequence. What kind of conditions, internal or external, are needed for the worms to swing and how does one partner recognize its swinging counterpart? Photo- (*cf.* Goto and Yoshida, 1984) and mechano-senses (Horridge and Boulton, 1967; Fraser, 1969; Feigenbaum and Reeve, 1977) have been described, both behaviorally and morphologically. Since the simple pigment-cup eye of chaetognatha does not form an image, a vibration sense appears to be a candidate for partner recognition. The ranges of the vibration sense which has been revealed using the feeding response of starved worms as criteria are from 9 to 20 Hz in *S. cephaloptera* (Horridge and Boulton, 1967) and from 8 to 140 Hz in *S. schizoptera* (Feigenbaum and Reeve, 1977). Careful observations of satiated worms under low frequency vibration is needed.

When two worms encounter each other, they touch one another with their heads (Fig. 1c) as if they were trying to estimate the distance between themselves. Similar behavior has been described in a closely related species, *S. cephaloptera* (John, 1933; Ghirardelli, 1968); however, the body alignment (Fig. 1d) followed by the unusual copulation observed in *S. schizoptera* (Fig. 1e) has not been reported.

Cannibalism is common in this species. Interestingly, the worms that begin to swing are never cannibalized; they concentrate on mating.

According to Vasiljev (1925) and Ghirardelli (1953, 1968), mating specimens of *S. cephaloptera* align in parallel, taking a head-to-tail position and simultaneous sperm transfer occurs. Vasiljev (1925) states that sperm are directly placed on the genital orifice whereas Ghirardelli (1953, 1968) observed that sperm are first placed on the neck region and later move toward the genital orifice. In *S. schizoptera*, paired sperm clusters (Fig. 2) are transferred non-reciprocally from one animal to a point near the genital orifice of another animal (Figs. 4 and 5a). The transferred sperm appear to move through the genital orifice (arrow in Fig. 5b) toward the seminal receptacles.

These observations suggest that for successful sperm transfer, three requirements have to be fulfilled: (1) the face-to-face alignment of partners, (2) the partners must be the appropriate distance from each other, and (3) the proper orientation during jumping and hitting must be achieved. Improper placement of sperm clusters was occasionally observed, due perhaps to the failure of completing one of the above steps.

Ejaculation may be induced by the mechanical stimuli of the hitting action at the time of sperm transfer. This is supported by the fact that light touching with a needle results in ejaculation (Fig. 3a, b). As described by Alvariño (1983b), the released sperm are transferred as a cluster (Fig. 5a, b) not in the form of a spermatophore as is true in arthropods and gastropods. Reeve and Cosper (1975) have suggested that the tightly packed sperm mass of *Sagitta hispida* is enveloped by a thin layer of material, presumably an adhesive mucoid. We did not observe such a layer with TEM or SEM.

It is not known how donorship is determined during mating in *S. schizoptera*. It is not evident whether the actively approaching worm becomes the donor. It is at least certain that a worm which releases its sperm cannot become a donor in the successive mating and that the maturity of the ovary is not related to the mating behavior. Also, sperm transfer is not an essential prelude for egg-laying, because isolated worms lay unfertilized eggs. This means that self-fertilization does not take place under natural conditions.

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MEIOTIC ARREST IN OOCYTES REGULATED BY A *SPISULA* FACTOR

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ABSTRACT

Ovarian oocytes of the mouse, *Spisula* and *Chaetopterus* are arrested in the dictyate stage of meiotic prophase. Upon isolation, mouse and *Chaetopterus* oocytes undergo spontaneous maturation manifested by germinal vesicle breakdown (GVBD) while *Spisula* oocytes retain their germinal vesicles. The present report describes a substance isolated from *Spisula* with meiotic arresting activity. The substance was purified from *Spisula* tissues by 70% ethanol extraction, chromatography on a Dowex 1-×8 column, and reversed phase HPLC.

GVBD in *Spisula* oocytes can be induced by insemination (5×10^4 sperm/ml) or treatment with serotonin or KCl. A crude ethanolic extract of *Spisula* tissues blocked oocyte maturation induced with serotonin. Upon washing in natural seawater (NSW) the oocytes proceeded to undergo maturation. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) blocked GVBD induced with sperm or serotonin. Dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) and dibutyryl cyclic guanosine 3′,5′-monophosphate (dbcGMP) blocked GVBD induced by sperm, while higher concentrations of these nucleotides were required to block serotonin-induced GVBD. However, none of the compounds tested including the *Spisula* extract influenced KCl-induced GVBD.

Isolated *Chaetopterus* oocytes suspended in artificial seawater (ASW) retained their GV and underwent spontaneous GVBD when placed in NSW. *Spisula* extract, dbcAMP and dbcGMP inhibited maturation of *Chaetopterus* oocytes suspended in NSW. dbcGMP, however, was a more potent inhibitor than dbcAMP, suggesting that cGMP may be the factor that maintains meiotic arrest in *Chaetopterus* oocytes. Spontaneous maturation of mouse oocytes was blocked by HPLC-purified *Spisula* factor at a concentration of 5 μg/ml in combination with 50 μM dbcAMP. We conclude that a substance found in *Spisula* tissues sustains meiotic arrest in mouse, *Spisula*, and *Chaetopterus* oocytes.

INTRODUCTION

Female gametes develop to a specific stage of meiosis and remain arrested at that stage while in the ovary and in some species even after spawning (Kanatani, 1973; Tsafriri, 1978; Masui and Clarke, 1979; Channing *et al.*, 1980; Meijer and Guerrier, 1984). The factor(s) that maintains oocytes in the arrested state of prophase I of meiosis has not been identified. One of the arresting factors may be intracellular cyclic nucleotides (Dekel and Beers, 1978). This hypothesis is based on the findings that compounds that increase intracellular cyclic adenosine 3′,5′-monophosphate (cAMP), *e.g.*, cholera toxin, forskolin, dibutyryl cAMP (dbcAMP), theophylline and 3-isobutyl-1-methylxanthine (IBMX), also inhibit spontaneous or hormone-induced maturation

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of isolated oocytes (Maller *et al.*, 1979; Hubbard and Terranova, 1982; Powers and Paleos, 1982; Tsafriri *et al.*, 1982; Sato and Koide, 1984a; Cho *et al.*, 1974; Nekola and Smith, 1975; Stern and Wassarman, 1975; Hillensjo, 1977; Hillensjo *et al.*, 1978; Schorderet-Slatkine *et al.*, 1978). We have demonstrated that bovine follicular fluid contains substances that sustain meiotic arrest (Sato and Koide, 1984b). Evidence will be presented showing that a substance isolated from *Spisula* sustains meiotic arrest in mouse, *Spisula*, and *Chaetopterus* oocytes.

Materials and Methods

The present investigation was conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts, during July and August of 1983 and 1984. Specimens of *Spisula soldissima* and *Chaetopterus pergamentaceus* were obtained from the Department of Marine Resources. Prepubertal Swiss mice (Nelson-Collins strain) of approximnately 15–20 g body weight were obtained from the animal facility at The Rockefeller University, New York.

Chemicals

Forskolin (HL 362, lot no. RC 1622) was a gift of Hoechst-Roussel Pharmaceuticals Inc., New Jersey. IBMX, serotonin (5-hydroxytryptamine hydrochloride), dbcAMP, dibutyryl cyclic guanosine 3′,5′-monophosphate (dbcGMP), and other chemicals were purchased from Sigma Chemicals.

Preparation of oocytes and sperm

Ovaries from female *Spisula* and *Chaetopterus* were excised, minced in artificial seawater (ASW), and strained through a pad of cheesecloth into a large beaker containing ASW (Cavanaugh, 1974). The oocytes were allowed to settle by gravity and the suspension medium was aspirated off. This washing process was repeated at least three times. Suspensions of oocytes prepared in this manner were used within one hour after extirpation. Excised testes from *Spisula* were stored in the refrigerator until used. During the storage period, a milky seminal fluid containing sperm was collected. This sperm suspension was drawn into calibrated pipettes, to permit accurate measurement of sperm concentrations.

Mouse oocytes were obtained as described in a previous paper (Sato and Koide, 1984a).

Induction of germinal vesicle breakdown (GVBD) of Spisula *and* Chaetopterus *oocytes*

One drop of a suspension containing approximately 2000 *Spisula* oocytes was added to 5 ml of ASW contained in a Falcon tissue culture dish (55 mm in diameter). GVBD of the *Spisula* oocytes was induced by insemination (final concentration of sperm: 8×10^6/ml), or by treatment with KCl (35 mM) or serotonin (50 μM). GVBD of *Chaetopterus* oocytes was induced by transfer into NSW. After 30 min, oocytes were examined under a dissecting microscope to determine the presence or absence of germinal vesicles. At least 500 oocytes were scored for each determination.

Mouse oocytes

Oocytes were cultured immediately after recovery in an incubator continuously flushed with an atmosphere of 95% air and 5% CO_2 saturated with water at

37 ± 0.5°C. About 10 to 15 oocytes were pipetted into 0.2 ml of medium under light paraffin oil. After 3 h of incubation, the oocytes were scored for the presence or absence of intact germinal vesicles.

Purification of a meiosis arresting factor from Spisula *tissues*

Spisula tissues (muscles and gonads) were excised, cut into small pieces, suspended in 70% ethanol, and homogenized in a Waring blendor for 5 min. The resulting brei was left standing at 4°C overnight, and filtered. The ethanolic extract (500 ml) was concentrated in a rotary vacuum evaporator at 38°C. The concentrated extract was dialyzed against 1 liter of distilled water at 4°C overnight, twice. The diffusate was applied to a Dowex 1-×8 resin column (6 × 10 cm) (200–400 mesh, chloride form, Bio-Rad) and eluted in sequence with 500 ml each of H_2O, 0.005 *N* HCl and 750 ml of 0.1 *N* HCl. The pooled samples were concentrated to 50 ml and lyophilized. The lyophilized powder was dissolved in 50 ml of water, neutralized with 1 *N* NaOH to pH 6.5 and lyophilized. The dry powder was dissolved in culture medium and assayed for activity. The active fraction located within the 0.1 *N* HCl fraction was further purified by high performance liquid chromatography using a Waters Radial Pak column. The column was eluted with 0.05 *M* $NH_4H_2PO_4$, and 10% aqueous methanol, pH 5.0, at a flow rate of 2.0 ml/min.

Assay for maturation inhibitory activity

The crude ethanol extract of *Spisula* tissues, and the oviducts purified by chromatography on Dowex 1-×8 and reversed phase HPLC were tested for GVBD inhibitory activity with oocytes of *Spisula, Chaetopterus,* and the mouse. The test samples were added to the suspension medium at varying concentrations. The percent inhibition was calculated according to the following formula:

$$\%\ \text{inhibition} = \frac{\%\ \text{oocytes GVBD (control)} - \%\ \text{oocytes GVBD (expt)}}{\%\ \text{oocytes GVBD (control)}} \times 100.$$

RESULTS

Spisula *oocytes*

The ethanolic extract of *Spisula* tissues at concentrations of 1 mg/ml or higher inhibited oocyte maturation induced with serotonin (Fig. 1). However, KCl-induced maturation was not affected. It was further observed that *Spisula* muscle extract at 1 mg/ml or higher suppressed motility of *Spisula* sperm. Because of the inhibitory influence on sperm motility, we were unable to evaluate the effect of *Spisula* muscle extract on sperm-induced GVBD of *Spisula* oocytes.

Spisula oocytes, induced by serotonin (5 μM) underwent GVBD within 15 min. (Fig. 2). After 30 minutes a gradual increase in the frequency of GVBD had occurred and by 30 min about 78% of oocytes had undergone GVBD. When oocytes were incubated in a medium containing *Spisula* extract at a concentration of 3 mg/ml, marked inhibition of GVBD resulted, *i.e.*, 88% of oocytes retained intact GV after 30 min of incubation (Fig. 2). When the treated oocytes were transferred to a control medium, 70% underwent GVBD within 15 min (Fig. 2). Hence, the inhibition affected by *Spisula* extract is reversible.

As control substances, forskolin and IBMX were tested. At concentrations of 5 μg/ml and higher both compounds inhibited dramatically the maturation of oocytes

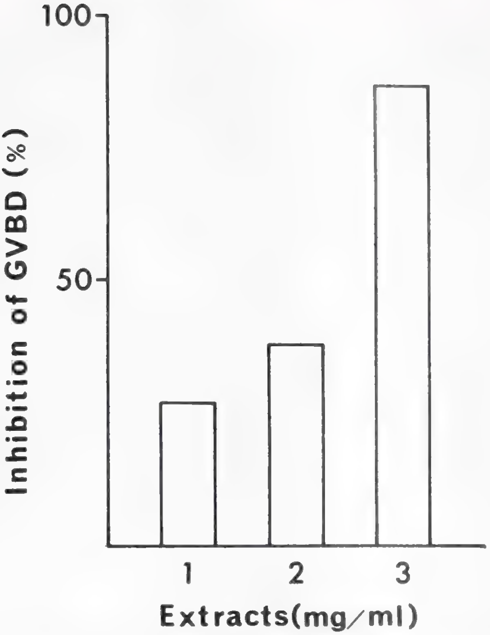

FIGURE 1. Effect of *Spisula* factor on the maturation of *Spisula* oocytes incubated with serotonin (5 μM). A lyophilized 70% ethanol extract of *Spisula* tissues was used.

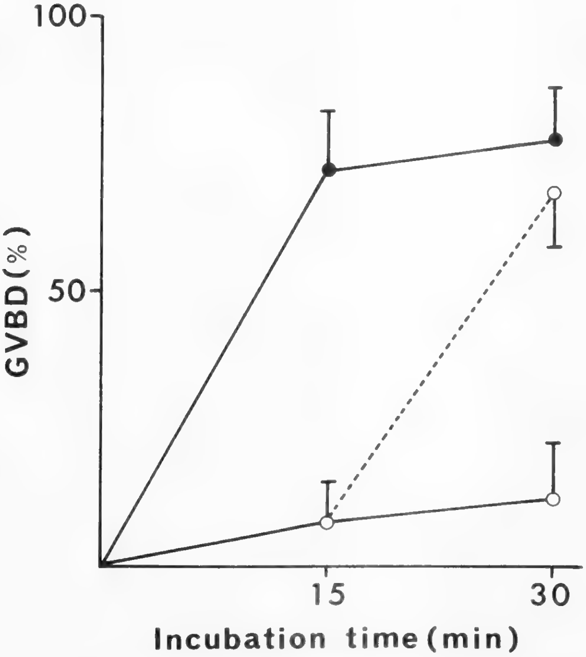

FIGURE 2. Effect of *Spisula* extract on the time course of GVBD of *Spisula* oocyte induced with serotonin (5 μM). ●——●, oocytes incubated in control medium, ○——○, incubated with *Spisula* extract, ○ ----- ○, after 15 min of treatment, the oocytes were washed in ASW three times, and transferred to control medium.

induced by sperm and serotonin (data not shown). However, KCl-induced maturation was not affected. dbcAMP at concentrations of 0.1, 0.5, and 1.0 m*M*, exerted 28, 68 and 70% inhibition of GVBD induced with sperm, and 0, 11, and 24% inhibition induced with serotonin, respectively. Again, KCl-induced GVBD was not affected by dbcAMP (data not shown). dbcGMP at the concentrations used had a slight inhibitory effect on sperm-induced GVBD, while GVDB-induced with KCl or serotonin was not affected.

Chaetopterus *oocytes*

When the crude ethanol extract of *Spisula* tissues was assayed, it blocked maturation of *Chaetopterus* oocytes suspended in NSW (Fig. 3). Inhibition of GVBD was 13, 68, and 79% at concentrations of 1, 2 and 3 mg/ml, respectively.

As control compounds, several nucleotides were tested for their ability to block GVBD in *Chaetopterus* oocytes suspended in NSW. GVBD was inhibited in 18 and 46% of oocytes with dbcAMP at concentrations of 1.0 and 2.0 m*M*, respectively (data not shown). Inhibition of GVBD with dbcGMP occurred in 46, 84, and 96% of oocytes at concentrations of 0.5, 1.0, and 2.0 m*M*, respectively. The following compounds had no effect on GVBD: adenine, adenosine, adenosine 5′-monophosphate, adenosine 2′,5′-diphosphate, adenosine 5′-diphosphate, adenosine 2′,3′-cyclic monophosphate, adenosine 2′,3′-cyclic phosphate 5′-monophosphate, adenosine 3′,5′-diphosphate, 8-bromoadenosine 3′-5′-cyclic monophosphate, guanosine 3′,5′-cyclic monophosphate, and guanosine 5′-triphosphate.

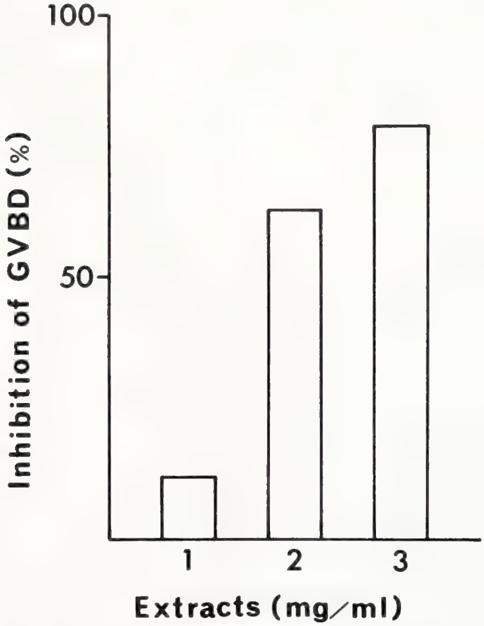

FIGURE 3. Effect of the *Spisula* factor on the maturation of *Chaetopterus* oocytes in NSW. Oocytes were examined for GV after 30 min suspension in NSW. A lyophilized 70% ethanol extract of *Spisula* tissues was used.

Mouse oocytes

Spisula extract was further purified by chromatography on a Dowex 1-×8 column. It was assayed for its ability to prevent GVBD in isolated cumulus-free mouse oocytes (Fig. 4). The GVBD inhibiting activity was found in the 0.1 *N* HCl fraction eluted from the Dowex column. At the third hour of incubation, 78% of the control oocytes had undergone GVBD. dbcAMP (50 μM) and the 0.1 *N* HCl fraction (2.5 mg/ml) added individually to the medium had a slight inhibitory effect. However, when varying concentrations of 0.1 *N* HCl Dowex fraction of the *Spisula* substance were coupled with 50 μM dbcAMP significant inhibition of GVBD occurred. GVBD was prevented in 47 and 59% of oocytes at concentrations of 1.0 and 2.5 mg/ml of *Spisula* substance (0.1 *N* HCl Dowex fraction), respectively.

The *Spisula* substance separated on the Dowex column was further purified by HPLC (Fig. 5). Two major peaks were eluted. The second peak possessed the GVBD inhibiting activity. HPLC-purified *Spisula* factor tested alone at concentrations of 5 and 10 μg/ml did not influence the spontaneous maturation of mouse oocytes (Fig. 4). When added with dbcAMP (50 μM) the frequency of GVBD of isolated mouse oocytes was blocked. Inhibition was 68 and 85% at concentrations of 5 and 10 μg/ml, respectively.

DISCUSSION

Compounds that increase the intracellular cAMP level or derivatized cAMP such as cholera toxin, forskolin, dbcAMP, theophylline, and IBMX inhibit maturation of oocytes, suggesting that the arrest of meiosis at the dictyate state may be regulated by intracellular cyclic nucleotides (Dekel and Beers, 1978; Sato and Koide, 1984a). The present results obtained with *Spisula* and *Chaetopterus* oocytes support this thesis. One distinct difference between the oocytes of *Spisula* and *Chaetopterus* has been the

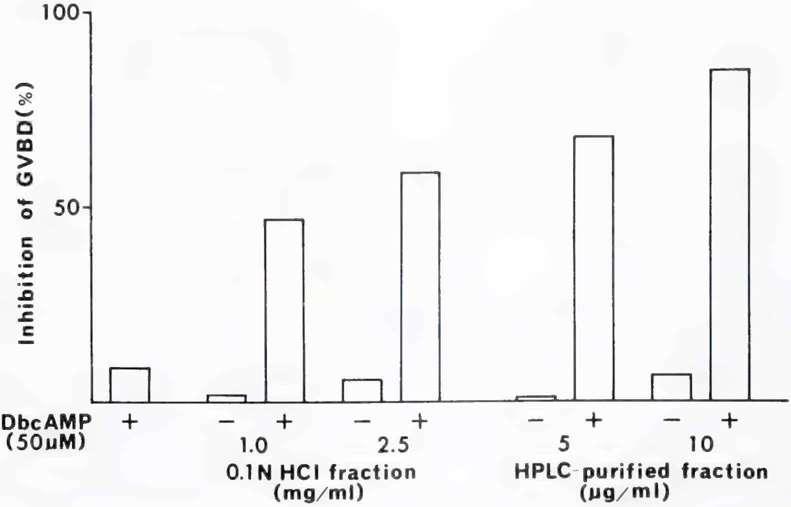

FIGURE 4. *In vitro* effect of purified *Spisula* factor on GVBD of isolated mouse oocytes. Spisula factor was purified by chromatography on a Dowex 1-×8 column and by high performance liquid chromatography on Waters Radial Pak column (Fig. 5).

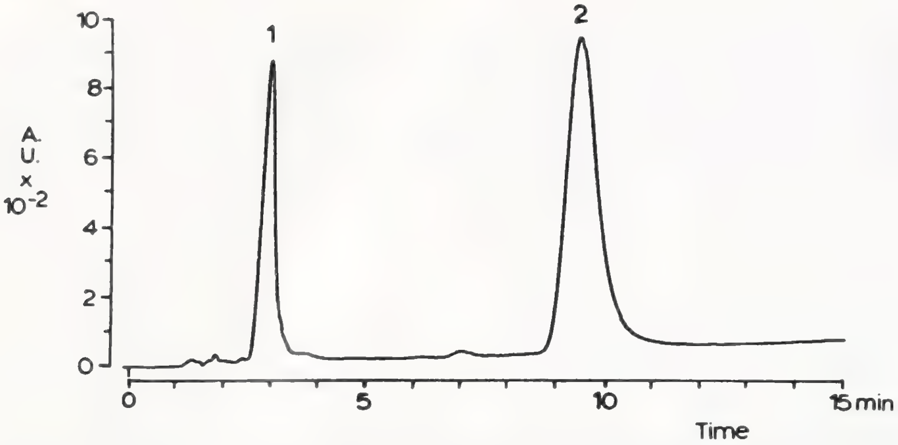

FIGURE 5. Elution pattern of *Spisula* factor purified by high performance liquid chromatography. Conentrated sample of the 0.1 *N* HCl fraction obtained from Dowex 1-8× column was applied to a Water Radial Pak column, eluted with 0.05 *M* $NH_4H_2PO_4$ in 10% aqueous ethanol, pH 5.0, at a flow rate of 2 ml/min.

effectiveness of cAMP *versus* cGMP. In *Spisula* oocytes dbcAMP was a very potent inhibitor of GVBD when induced with sperm or serotonin. In *Chaetopterus,* dbcGMP was the more potent suppressor of GVBD. It was reported that both dbcAMP and dbcGMP can arrest mammalian oocytes at the dictyate stage except that the suppressive activity of dbcAMP was more pronounced than dbcGMP (Hubbard and Terranova, 1982). dbcGMP may exert its inhibitory effects through the cumulus cells, while dbcAMP appears to act directly on the oocytes (Hubbard and Terranova, 1982). The observed variation in sensitivity of oocytes of different species to dbcAMP and dbcGMP deserves further attention.

It was demonstrated that the *Spisula* factor alone blocked GVBD in *Spisula* and *Chaetopterus* oocytes. Inhibition is induced only when combined with dbcAMP in mouse oocytes. The present results suggest that the *Spisula* factor may be an important meiotic arresting substance. Experiments are being undertaken to determine the chemical structure of the *Spisula* factor.

An inhibitor of mouse oocyte maturation was demonstrated to be present in porcine follicular fluid which acts synergistically with dbcAMP (Downs and Eppig, 1984). This compound was identified as hypoxanthine (Downs *et al.*, 1985). The *Spisula* factor can be separated from hypoxanthine by chromatography on Dowex 1-8× column and by reversed phase HPLC.

GVBD can be induced in *Spisula* oocytes with sperm, KCl, and serotonin (Allen, 1953; Hirai *et al.*, 1984). Hirai *et al.* (1984) demonstrated that *Spisula* oocytes treated with KCl or serotonin produced maturation promoting fator (MPF). Since the *Spisula* factor blocked serotonin-induced GVBD and not KCl-induced GVBD, the site of inhibition is probably prior to the production of MPF. This contention is further supported by the findings that GVBD induced with KCl is not inhibited by dbcAMP, while GVBD induced with sperm and serotonin are blocked. Thus, the *Spisula* factor and cAMP analogs appear to block the formation of MPF rather than its action.

The *Spisula* factor also suppressed the forward motility of *Spisula* sperm. Within 2 or 3 min after incubation at concentrations of 1 and 2 mg/ml, sperm became immobile. The *Spisula* factor is not cAMP since this nucleotide stimulates sperm motility (Hoskins *et al.*, 1974, 1975; Acott and Hoskins, 1978; Acott *et al.*, 1979).

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OVULATION AND THE FINE STRUCTURE OF THE *STICHOPUS CALIFORNICUS* (ECHINODERMATA: HOLOTHUROIDEA) FECUND OVARIAN TUBULES

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ABSTRACT

The ovary of *Stichopus californicus* consists of several size classes of tubules, which insert into a central gonad basis. The largest tubules contain the oocytes that will be spawned in the current season. All tubules are composed of three layers. Outermost is a complex peritoneum composed of epithelial cells, axons and muscle cells. The fine structure of the peritoneal neurons suggests their involvement in neurosecretory activity. Between the basal laminae of the peritoneum and the inner epithelium is the ovarian connective tissue compartment, including the genital hemal sinus. This sinus probably conveys nutrients from the periphery of the tubule to oocytes located deep within. The inner epithelium is composed of parietal and follicular epithelial cells and the oocytes. *Stichopus* oocytes contain three classes of microtubules based upon their location, orientation, and lability during fixation. Microtubules from the apical protuberance encircle the germinal vesicle. Cortical microtubules lie just under the cell surface and run parallel to it. Deep cytoplasmic microtubules run radially from the interior of the oocyte towards the cell surface. Oocytes are held within follicles by junctional complexes until the time of ovulation. Ovulation can be monitored in severed follicles of this species because an oolamina insures follicle integrity after detachment from the ovary. The onset of ovulation is marked by the dissolution of junctional complexes. This is followed by a cytochalasin B sensitive contraction of the follicle cells. The follicle contracts down around the oocyte, to lie collapsed against the ovarian wall while the oocyte is free within the ovarian lumen.

INTRODUCTION

Ovulation in the aspidochirote holothurian *Stichopus californicus* (Stimpson, 1857) involves the extrusion of oocytes from epithelial follicles during normal spawning. Ovulation may be experimentally induced when excised ovaries or severed follicles are exposed to seawater. In the process of ovulation, junctions between oocytes and follicular epithelial cells break. This must occur before the oocytes are released and can subsequently mature. Ovulation has been described for asteroids (Schroeder, 1971; Schroeder *et al.*, 1979) and a crinoid (Holland and Dan, 1975), but little is known for holothurians. Study of the process is facilitated in this holothurian because ovulation will occur spontaneously in isolated ovarian fragments, and the ovulation of individual oocytes can be easily followed under the microscope. Analysis of holothurian ovulation must include a detailed understanding of the structure of the ovary and especially of the ovarian inner epithelium.

The anatomy and histology of the holothurian ovary is summarized in the major treatises of invertebrate zoology (Ludwig, 1889–1892; Cuenot, 1948; Hyman, 1955).

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More detailed accounts of individual species appear in monographs (Gerould, 1896; Theel, 1901; Ohshima, 1921; Inaba, 1930; Menker, 1970). The holothurian ovary is an unpaired organ attached to the dorsal mesentery. It consists of a thick, fleshly central gonad basis and numerous blind ending tubules. The gonoduct exits the gonad basis from its dorsal anterior aspect and runs within the mesentery to the gonopore.

Holothurian oocytes are surrounded by a layer of squamous inner epithelial cells, called follicle cells. Ludwig (1889–1892) described a stalk supporting the oocytes in the aspidochirote holothurian *Holothuria mamorata*, but did not ascertain if the stalk was formed from follicle cells. Gerould's (1896) description of an intricate connection between the oocyte and follicle cells in the molpadiid holothurian *Caudina arenata* makes it clear that further investigation of these cells is required. Davis (1971) analyzed the gonad histology of a number of different echinoderm species using transmission electron microscopy. The focus of her study did not include a fine structural analysis of the inner epithelium of these species, although she did examine the structure of the ovarian wall of *Stichopus californicus*. There have been no electron microscopic analyses of the cellular relationships in the ovarian inner epithelium of a holothurian, nor are there reports of ovulation in holothurians or any experimental analysis of the process of ovulation.

This paper presents a detailed description of the fine structure of the ovarian fecund tubules in *Stichopus californicus*, and an experimental analysis of the process of ovulation. Our investigations corroborate the published descriptions of the general organization of the ovary and present new information on the cellular relationships within the inner epithelium of the fecund tubules. Our observation of three classes of microtubules within this oocyte may pertain to the expression of oocyte polarity. We extend previous descriptions of the fine structure of the investing peritoneum and connective tissue compartment, containing the genital hemal sinus. We also confirm reports of the complete resorption of the fecund ovarian tubules after spawning (Theel, 1901; Tyler and Gage, 1983). These results are discussed in light of the twin functions of the holothurian ovary, oogenesis and spawning, and compared with available descriptions of other echinoderms. The architectural simplicity and the primitive character of the holothurian ovary suggest that the fine structural descriptions presented here will be of value in understanding structure and function in other echinoderm ovaries.

MATERIALS AND METHODS

Specimens of *Stichopus californicus* were collected by dredging or by diving, and maintained in running seawater at The Friday Harbor Laboratories of the University of Washington. Ovaries were excised from healthy animals and fixed either in phosphate buffered glutaraldehyde (Cloney and Florey, 1968) or in a cacodylate buffered cocktail fixative (Eakin and Brandenburger, 1979). Osmication followed primary fixation and was done with the appropriate buffer. Isopropyl alcohol or acetone were used for dehydration. Propylene oxide was used as the antemedium before infiltration and embedment in Epon. One micron thick sections were stained with a mixture of azure II and methylene blue. Silver or silver-gold thin sections mounted on copper grids, stained with lead citrate and a solution of saturated uranyl acetate and methanol were viewed on a Philips EM 300 or 301 electron microscope. Glyoxilic acid staining for catecholamines was done by the method of Burke (1983).

Stichopus oocyte microtubules are not preserved when fixed in phosphate buffers. Microtubules of the protuberance are preserved with the cacodylate buffered cocktail fixative at room temperature and when the pH is 7.2. Preservation of cortical microtubules is enhanced when the temperature of this fixative is kept below 4°C. The deep cytoplasmic microtubules are preserved with this fixation at 4°C and the pH of the buffer is between 7.5 and 7.8. We fixed portions of ovaries of more than 25 *Stichopus* individuals with each of these fixation regimes, then sectioned and examined them to determine the reliability of these fixations in preserving the different classes of oocyte microtubules. In all cases, microtubules are best preserved in oocytes that remain within the ovary.

Ovulation can be easily studied in *Stichopus californicus* because the entire process occurs spontaneously when the fecund tubules are torn open in sea water, and most oocytes ovulate quickly. Contraction of arrays of thin filaments has been implicated in a number of cellular movements (Cloney, 1966; Schroeder, 1972). Cytochalasin B disorganizes arrays of actin thin filaments and stalls their contraction (Schroeder, 1972, Wessels *et al.*, 1971). We used cytochalasin B prepared from a 1 mg/ml stock solution dissolved in dimethyl-sulfoxide (DMSO) in testing our hypothesis that actin filaments were involved in ovulation. The drug was diluted to 10 μg/ml with filtered seawater in the experiments. Controls containing 1% DMSO in filtered seawater had no effect on ovulation.

Stichopus californicus (Clark, 1922), hereafter called *Stichopus,* has also been referred to as *Parastichopus californicus* (Deichmann, 1937).

RESULTS

Anatomy of the gonad

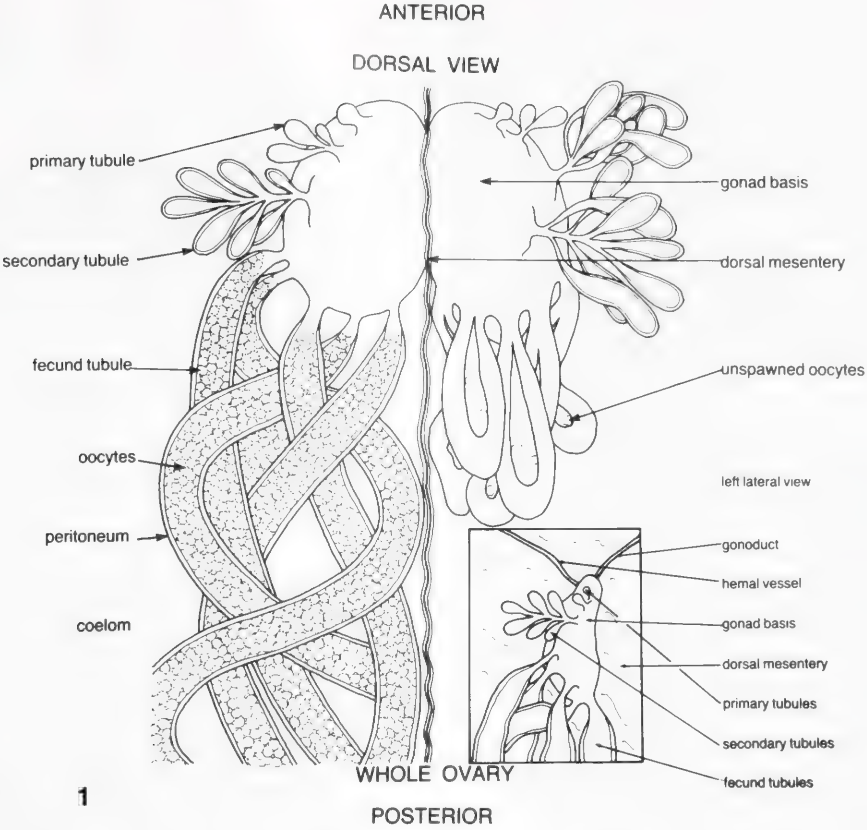
The ovary of stichopodid aspidochirote holothurians is a single bilaterally symmetrical organ; it consists of a gonad basis, numerous fecund tubules, and inconspicuous unripe tubules (Fig. 1). The gonad basis is a fleshy thickening in the dorsal suspensor mesentery to which all tubules attach. The tubules inserting on the flanks of the basis increase in size from anterior to posterior (Fig. 1). The largest, most posterior tubules are fecund before spawning and contain post-vitellogenic oocytes, 185–200 μm in diameter. In the late spring these tubules nearly fill the perivisceral coelom. The number of fecund tubules on the basis is variable, but there are usually between 10 and 12, and each branches dichotomously many times along its length. The aggregate lineal dimension of all the branches in a single tubule is about 38 cm. Fecund tubules average 3.1 mm in diameter but become thinner near their insertion on the basis. The volume of all the fecund tubules is about 185 ml during the late spring, close to the time of spawning.

Structure of the fecund tubules

Fecund tubules of *Stichopus* have three layers, as determined by electron microscopy (Fig. 2). Outermost is a complex peritoneum continuous with the peritoneum that lines the perivisceral coelom. This complex peritoneum includes circular muscles and nerve fibers. A connective tissue compartment lies between the peritoneum and the inner epithelium. The connective tissue compartment contains the genital hemal sinus of the ovary. Longitudinal folds in the inner epithelium may extend several centimeters along the length of each tubule.

The outer layer of the ovary: the peritoneum

Epithelial cells. The peritoneum is composed of squamous to low cuboidal epithelial cells that are joined by apical zonulae adherentes and subjacent septate junctions (Fig. 3). The apical surface of these epithelial cells characteristically bears a single


ANTERIOR
DORSAL VIEW
primary tubule
secondary tubule
fecund tubule
oocytes
peritoneum
coelom
gonad basis
dorsal mesentery
unspawned oocytes
left lateral view
gonoduct
hemal vessel
gonad basis
dorsal mesentery
primary tubules
secondary tubules
fecund tubules
WHOLE OVARY
POSTERIOR

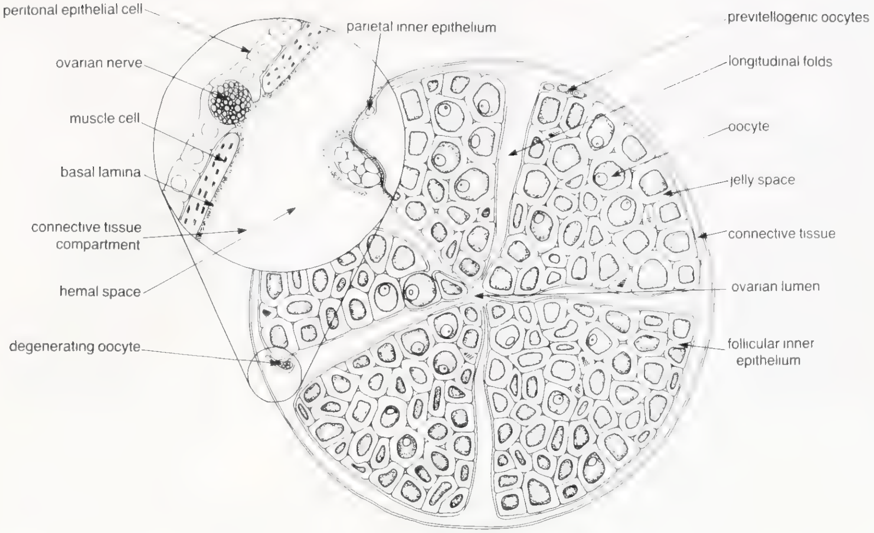
1

FIGURE 1. Whole ovary of *Stichopus californicus*. The left side represents the condition in the ovary prior to spawning, while the right side represents the post-spawning condition. The inset in the lower right depicts a left lateral view of the unspawned ovary.

cilium. Microvilli form a collar around the cilium and other microvilli cover the apical coelomic surface. Peritoneal epithelial cells contain dense arrays of rough endoplasmic reticulum that are uniformly distributed around the central nucleus. The epithelial cells often contain secondary lysosomes about 1.0 μm in diameter. The basal surface of the epithelial cells is associated with a basal lamina. Occasionally, hemidesmosomes are seen in muscle cells adjacent to the basal lamina (Fig. 4).

Nerves. Nerves are abundant in the ovarian peritoneum (Fig. 5). Each nerve is overlain by extensions of the peritoneal epithelial cells. Nerves are 2 to 4 μm in diameter and contain 25 to 250 axons. Assuming an average of 125 axons per nerve, 50 μm between nerves around the circumference of the tubule, and an average tubule circumference of 7 mm, the total number of axons in a single fecund tubule is approximately 24,000.

Axons are about 0.1 μm in diameter and contain microtubules, mitochondria, clear core vesicles about 50 nm in diameter, and dense core vesicles 80 to 150 nm in diameter. Some axons contain large vesicles 200 to 400 nm in diameter. Axon termini are enlarged to about 0.5 μm in diameter where the larger inclusions are found. Occasionally, aggregations of these large moderately staining vesicles are found in axons close to the hemal space (Fig. 5). No ovarian neuronal perikarya have been sectioned,


peritonal epithelial cell
ovarian nerve
muscle cell
basal lamina
connective tissue compartment
hemal space
degenerating oocyte
parietal inner epithelium
previtellogenic oocytes
longitudinal folds
oocyte
jelly space
connective tissue
ovarian lumen
follicular inner epithelium
CROSS SECTION
FECUND TUBULE

2

FIGURE 2. Cross-section of a fecund ovarian tubule illustrating the relative positions of the tissues. The inset circle represents a magnification of the smaller circle and depicts the organization of the complex peritoneum.

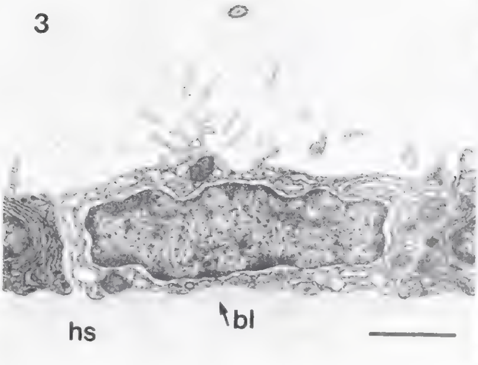
however, glyoxylic acid staining reveals that cell bodies containing reactive catecholamines are present in the outer layer of fecund tubules (Fig. 6). Neuromuscular junctions, membrane densitities, and gap junctions between axons and muscle cells have not been found. Because the nerve processes and the neuronal perikarya are superjacent to the peritoneal basal lamina we consider these nervous system elements part of this complex peritoneum.

Muscle cells. Light microscopy reveals a layer of muscle cells under the peritoneum in the ovarian tubules of *Stichopus.* However, electron microscopic examination of these muscle cells show they are also superjacent to the basal lamina of the peritoneum and lack an external lamina (Fig. 4). We infer that these muscle cells are myo-epithelial. The peritoneal muscle cells of the ovary form only a circular layer. Muscle cells occur every 20 μm along a tubule. A single tubule having an average length of 38 cm may, therefore, contain about 18,000 muscle cells.

Muscle cells nearly encircle the tubule, a distance of about 7 mm. Each appears to have a terminal perikaryon, but the position of the perikaryon is probably variable. Arrays of thick and thin filaments are similar to those in the smooth muscle of other echinoderms (Cavey and Wood, 1981). The diameter of thicker filaments is 50–65 nm, while that of the thinner filaments is 6–10 nm (Fig. 4). There is a reduced sarcoplasmic reticulum adjacent to the peritoneal basal lamina and mitochondria usually lie on the peritoneal side of the contractile elements. Desmosomes connect closely apposed muscle cells and dense plaques similar to hemidesmosomes occur on the basal surface of the cells. We found no gap junctions between muscle cells (Fig. 4).

Middle layer: the connective tissue compartment

The connective tissue compartment of the ovary lies between the basal laminae of the peritoneum and the inner epithelium of the ovary. It contains ground substance, 3



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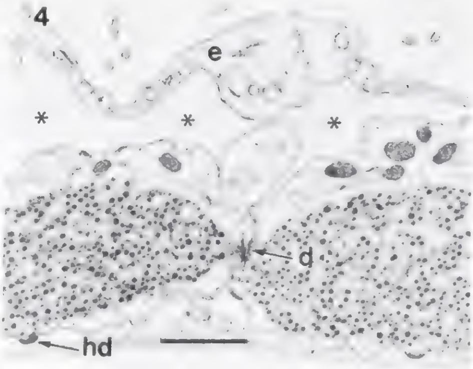

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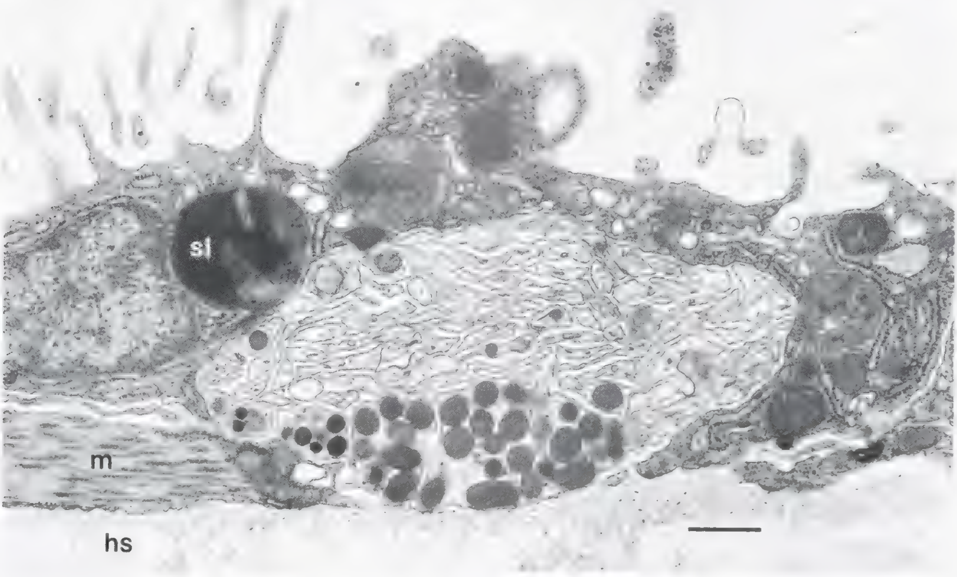


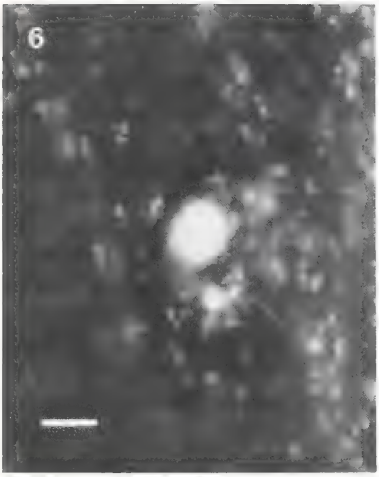
FIGURE 3. TEM of epithelial cells of the peritoneum. These cells lie on a basal lamina (bl) that separates them from the genital hemal sinus (hs) of the ovary. These cells often contain regular arrays of RER. Scale bar = 0.5 μm. 23,000×.

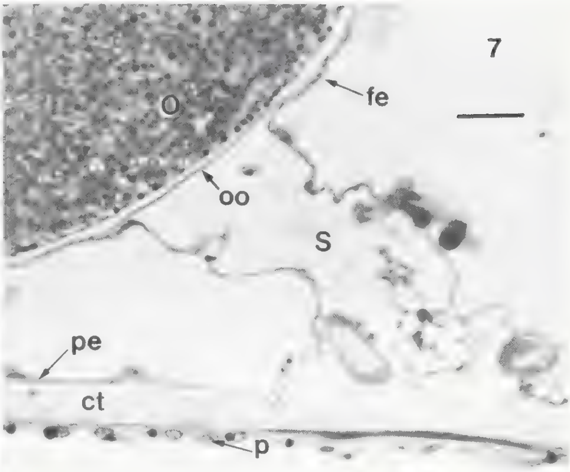
FIGURE 4. TEM of adjacent muscle cell contractile elements joined by a desmosome (d). The cells are attached to the basal lamina by hemidesmosomes (hd). Processes from epithelial cells (e) overlie the muscle cells. Spaces containing asterisks are fixation artifacts. Scale bar = 1.0 μm. 11,500×.

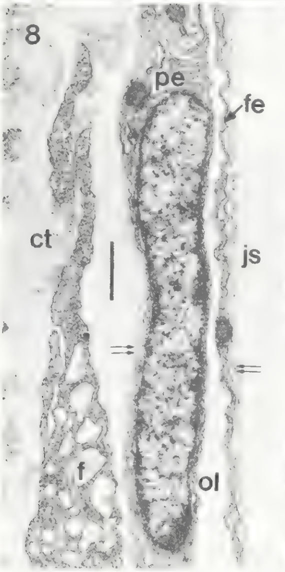
FIGURE 5. TEM of an ovarian nerve. Epithelial cells often bear secondary lysosomes (sl). Axons in this nerve lie on the basal lamina near the hemal space (hs) and contain large vesicles of moderate electron density. Muscle cells (m) underlie the epithelial cells and run perpendicular to the nerves. Scale bar = 0.5 μm. 19,500×.

fibers, fibroblasts, and the fluid filled sinus of the hemal system which carries some coelomocytes (Fig. 9).

Connective tissue fibers have the characteristics of collagen and the surrounding ground substance stains metachromatically with azure II. The fibers are about 35 nm in diameter and have a periodicity of about 65 nm (Fig. 8). The fibers are more densely aggregated near the basal lamina of the peritoneum than in other parts of the connective







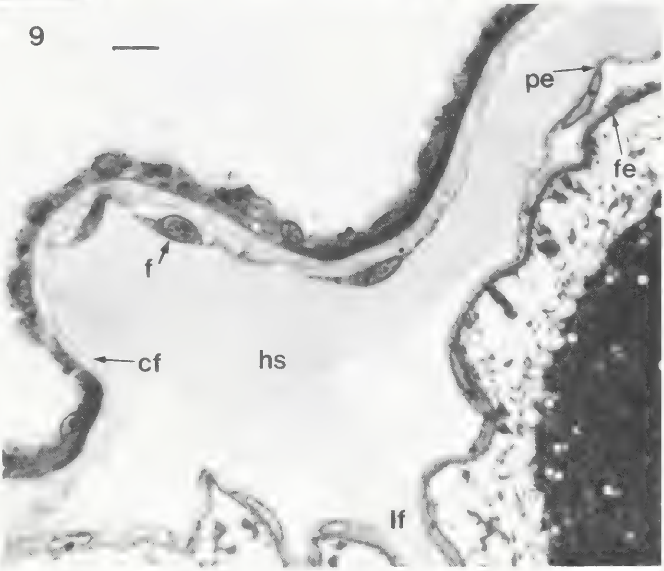


FIGURE 6. Glyoxylic acid induced catecholamine fluorescence of a putative neuronal perikaryon within the ovarian peritoneum. Scale bar = 10 μm. 710×.

FIGURE 7. Light micrograph (LM) of an oocyte (O) within its follicle. The follicular inner epithelium (fe) is continuous with the parietal inner epithelium (pe) at the stalk (S). The oocyte basal plate or oolamina (oo) separates the hemal sinus from the oocyte jelly space. The connective tissue compartment (ct), which includes the hemal sinus, spans the area between the peritoneal and inner epithelial basal laminae. Scale bar = 10 μm. 870×.

FIGURE 8. TEM of the closely applied apical surfaces of follicular (fe) and parietal (pe) inner epithelial cells. The basal laminae of these cells are indicated by double arrows. Within the connective tissues compartment (ct) is a fibroblast (f). The jelly space (js) surrounding the oocyte and the ovarian lumen (ol) are marked. Scale bar = 0.5 μm. 15,600×.

FIGURE 9. LM showing the organization of the connective tissue compartment with fibroblasts (f), fibers (cf), the serous hemal fluid within the hemal sinus (hs). The parietal (pe) and follicular (fe) inner epithelial cells are indicated, as well as the outermost part of a longitudinal fold (lf) in the inner epithelium. Scale bar = 10 μm. 620×.

tissue compartment. Fibroblasts usually occur among the aggregations of collagen fibers near the outer-most part of the connective tissue compartment of the fecund tubule (Fig. 9). Fibroblasts contain arrays of rough endoplasmic reticulum and bear long filopodial extensions, but their ultrastructure is otherwise unremarkable. Hemal fluid in the genital hemal sinus is a serous material that stains uniformly with Richardson's stain and appears flocculent in electron micrographs. Hemal fluid contains few cells; by far the most common are petaloid amoebocytes.

Innermost layer: the ovarian inner epithelium

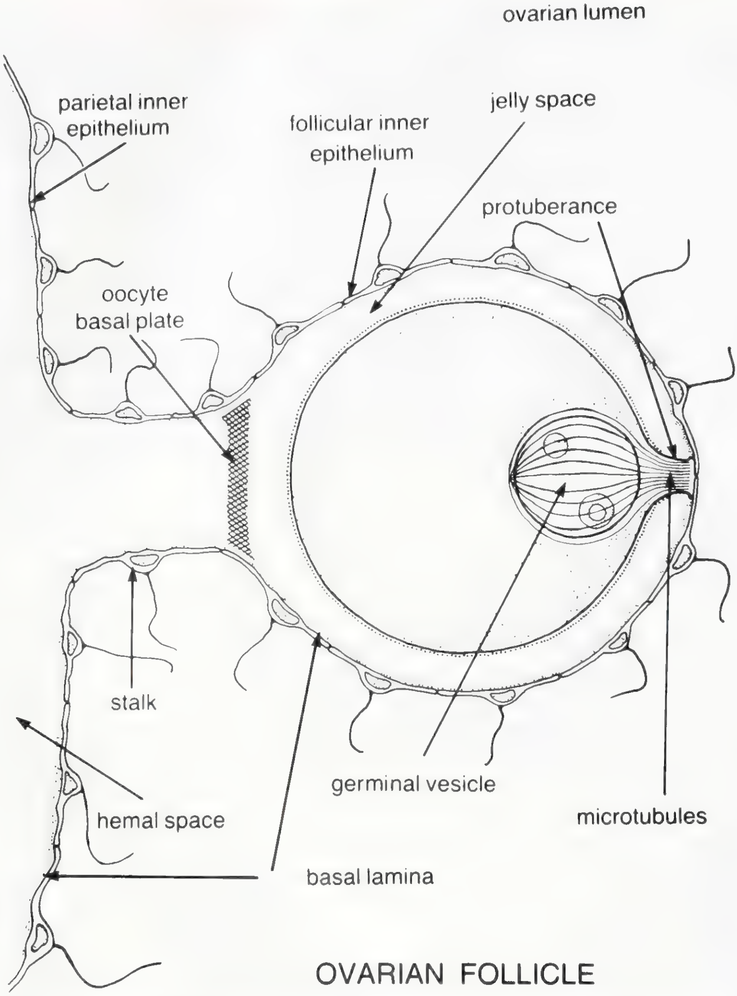
The inner epithelium of the fecund ovarian tubules of *Stichopus californicus* is composed of three cell types; parietal inner epithelial cells, follicular inner epithelial cells, and oocytes. Parietal inner epithelial cells form the inner lining of the ovarian tubules and follicular inner epithelial cells make up the cellular follicles surrounding the oocytes (Figs. 7, 8, 9). These somatic cell types are continuous with one another at the follicular stalk and separate the ovarian connective tissue compartment from the ovarian lumen. The distinction between parietal and follicular inner epithelial cells, while largely one of convenience, serves to distinguish those inner epithelial cells which form a follicle around the oocyte and may have some potent endocrine function, as is the case in asteroids (Hirai and Kanatani, 1971).

Somatic inner epithelial cells. The somatic inner epithelial cells are squamous, often less than 0.5 μm. in thickness (Fig. 8). The cells are thicker near the nucleus and near the single apical cilium. In the thickened parts of the cells, mitochondria and substantial numbers of microtubules occur but there are few Golgi bodies and little rough endoplasmic reticulum. A collar of microvilli surrounds the cilium but no additional microvilli are found on the apical surface of the cells. These cells lie on a basal lamina; they are interdigitated and joined by zonulae adherentes, but never by septate junctions (Fig. 11). The interdigitating processes often contain numerous minute vesicles.

Follicular inner epithelial cells form the follicles surround the oocytes of the inner epithelium. The arrangement of cells within a folicle is depicted in Figure 10. At the point where the follicular and parietal inner epithelial cells abut, near the base of the follicular stalk, an especially thickened basal lamina, the oocyte basal plate or oolamina, underlies the oocyte (Figs. 7, 12). An unusual 'T' shaped connection between the oolamina and the basal laminae of the somatic cells is found at the nexus of parietal inner epithelial cells, the follicular inner epithelial cells and the oolamina. The oolamina is considerably thicker than somatic basal laminae although its ultrastructure is similar (Fig. 12). The oolamina forms a barrier between the genital hemal sinus and the subfollicular jelly space surrounding the oocyte, allowing only restricted association of the hemal fluid with the oocyte surface.

Oocytes. Oocytes of *Stichopus californicus* are 185–200 μm in diameter just before spawning (Fig. 29). The oocyte has a prominent germinal vesicle about 80 μm in diameter which usually contains one or two vesiculated nucleoli from which densely staining threads radiate. Oocytes from fecund tubules are postvitellogenic and contain numerous membrane bound yolk granules and other inclusions.

Holothurian oocytes bear an axial protuberance referred to in the literature as a "microplye appendage" (Ludwig, 1889; Gerould, 1896; Ohshima, 1921; Inaba, 1930). The *Stichopus californicus* oocyte protuberance inserts into the follicle cell capsule at a point roughly opposite the follicular stalk and basal plate (Fig. 13). A zonula adherens occurs between the protuberance and the follicle cells at the point of insertion (Fig. 15). The follicle cells contain numerous vesicles in this region, which are about 0.1 μm in diameter and have a clear core.


ovarian lumen
parietal inner epithelium
follicular inner epithelium
jelly space
protuberance
oocyte basal plate
stalk
hemal space
germinal vesicle
microtubules
basal lamina
OVARIAN FOLLICLE

10

FIGURE 10. Relationships between the parietal and follicular inner epithelial cells and the oocyte. The oocyte basal plate or oolamina is the basal lamina of the oocyte.

The protuberance contains large numbers of microtubules which are more concentrated peripherally as determined by a comparison of medial and peripheral longitudinal sections (Figs. 14, 15). Polarization microscopy and TEM show that the microtubules of the protuberance extend into the ooplasm and surround the germinal vesicle (Fig. 17). These arrays of microtubules exclude granular inclusions from the protuberance and adjacent cytoplasm and produce striated zones in the oocyte near the animal pole (Fig. 13).

The oocyte cortex contains few bipartite granules reminiscent of those found in echinoids (Anderson, 1968). Differential interference contrast (DIC) microscopy of living oocytes reveals that the oocyte surface bears minute ridges or microplicae in addition to the microvilli (Fig. 21) which are best seen in Epon sections. Numerous microtubules are also found in the cortex under certain fixation regimes, but these are more labile than the microtubules of the protuberance (Fig. 16). Dense plaques occur on the outer surface of the oocyte plasma membrane. A few vesicles close to the plasma membrane also have dense plaques as part of their inner surface, suggesting that the oocyte plasma membrane is in flux with the plasma membrane of these vesicles.

Deep within the oocyte cytoplasm another kind of microtubule is found (Fig. 18). These microtubules are extremely labile to fixation and are preserved consistently only when the pH of the fixation fluid is between 7.5 and 7.8 and the fixation done on ice (see Materials and Methods). These deep cytoplasmic microtubules run radially from the interior of the cell toward the periphery. They are oriented perpendicular to cortical microtubules and are not connected to the protuberance.

The oocytes contain abundant mitochondria which are evenly dispersed throughout the cytoplasm. Golgi bodies, putative pigment vesicles, and yolk granules are also uniformly distributed in the oocytes (Fig. 16). Some of the yolk granules contain crystalloid cores (Fig. 23). Unusual spherical annulate lamellae (Fig. 22) are found in the oocyte cytoplasm. The germinal vesicle is unremarkable other than the association of the protuberance microtubules with the nuclear envelope.

A jelly space separates oocytes from the basal lamina on the inner surface of the follicle capsule. Two components with distinct tinctorial properties occur in the jelly space of oocytes ligated prior to fixation. These may represent jelly coat precursors. Oocytes from torn tubules, which have been exposed to seawater, bear fully formed jelly coats regardless of whether they have ovulated. Ovulated oocytes lose their jelly coats during the processing for embedment.

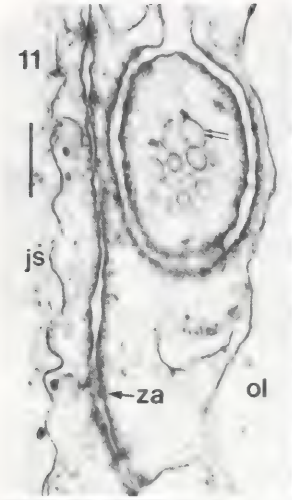
The ovarian lumen is restricted to the interstices between ovarian follicles in the fecund tubules; it sometimes contains petaloid amoebocytes (Fig. 20). The lumen of fecund tubules is directly connected to the gonoduct. Ovulated oocytes accumulate in the ovarian lumen until the time they are released from the gonoduct into the sea at spawning. Occasionally, both vesiculated and relict oocytes are found in the ovarian tubules (Figs. 19, 21). These are rare in freshly collected *Stichopus californicus* (Smiley, 1984).

Ovulation

Ovulation can be defined as the severance of the intimate cellular connections between an oocyte and its surrounding ovarian somatic cells. The first event in ovulation in *Stichopus californicus* is the dissolution of the adhering junctions between the follicle cells and the protuberance of the oocyte. This is manifested by the movement of the protuberance through the follicle (Fig. 25). A small annulus in the follicle surrounds the protuberance at this time. The annulus enlarges and the follicle cells appear to retract down around the oocyte. The follicle cells are separated from the oocyte surface during this process by the jelly coat. As the annulus in the follicle enlarges, the oocyte begins to squeeze through the opening (Fig. 26). This process is accompanied by a distortion of the oocyte, causing it to assume an hourglass-like shape (Figs. 26, 27). The rate of follicle recession increases as ovulation continues, and the final stages are quickly completed (Figs. 27, 28). After ovulation, oocytes lie free in the lumen, and the follicle cells remain attached to the ovarian wall (Fig. 29).

Ovulation can be induced in intact ligated fecund tubules by treating ligated tubules with a solution of asteroid radial nerve extract (Smiley, 1984). Ovulation commences about three hours after the extract is added, and proceeds similarly to that of oocytes isolated in their follicles. Peristalsis of the tubule musculature continues for about four hours after the addition of the extract, and ends when the ovulated oocytes are forced through a severed end of the tubule by strong contractions of the musculature.

One hypothesis accounting for the forces required for ovulation is that hydration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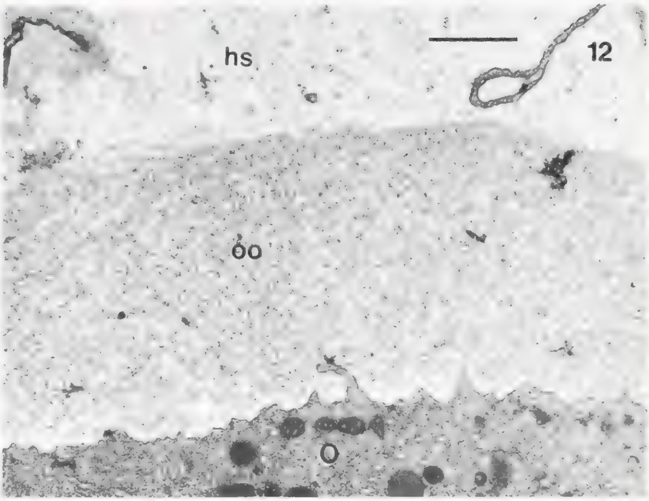
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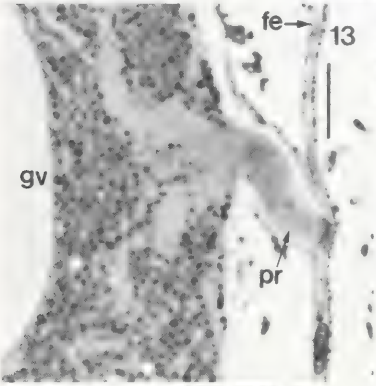
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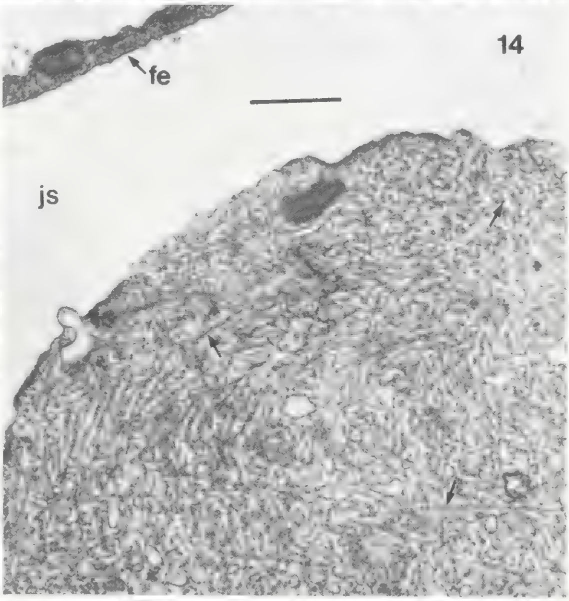
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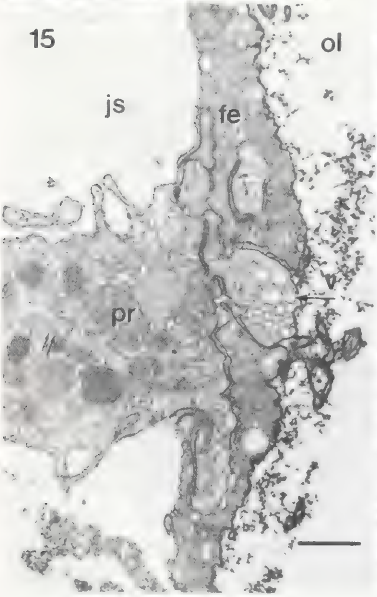
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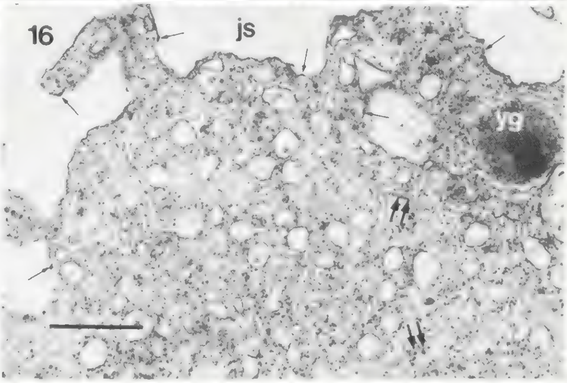
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of the jelly coat provides hydrostatic forces that push the oocyte out of the follicle. This explanation is attractive because the jelly coat is first visible about the time of ovulation. To test this hypothesis directly, tears were made in the follicles of unovulated oocytes with fine glass needles, and ovulation was monitored. The data from 50 such experiments, performed on oocytes from 6 different ovaries, are presented in Table I. It is clear that a tear in the follicle does not stop ovulation.

Electron microscopic examination of the follicle cells during ovulation reveals thin filaments within these cells (Fig. 26). The diameter of the filaments is between 6 and 8 nm suggesting that they may be actin filaments. Schroeder (1971), on the basis of ultrastructural analysis of asteroid follicles, concluded that contraction of arrays of actin filaments within these follicles was responsible for providing some of the force that drives ovulation in starfish. To test the hypothesis that contraction of actin filaments within the follicle cells provides some of the force required for successful ovulation of *Stichopus* oocytes, pieces of torn fecund tubules were placed in filtered seawater containing the drug cytochalasin B. Because ovulation can occur immediately after the fecund tubules are torn open, scoring was based on the number of oocytes that were stalled in ovulation after incubation for 30 minutes in the drug solution. Therefore, oocytes completing ovulation before the drug could act were not counted. Table II presents data from these experiments as numbers of oocytes stalled in ovulation after 30 minutes. Results from the cytochalasin B treatment of ovulating oocytes supports the hypothesis that at least a part of the force required for ovulation is provided by contraction of cytochalasin B sensitive actin filaments.

Spawning and tubule resorption

Ovulated oocytes are held within the tubule until the time of spawning. In uninduced spawns observed in aquaria, *Stichopus californicus* releases its oocytes as the germinal vesicles are breaking down. Based upon dissection of spawned animals from the field and the laboratory, spawning in *Stichopus californicus* is a catastrophic event which usually results in nearly complete evacuation of oocytes from fecund tubules. The release of oocytes considerably diminishes the size of the tubules, and virtually no normal oocytes remain in the tubules after spawning.

FIGURE 11. TEM of an interdigitation of follicle cells. Vesicles (double arrows) are found in the interdigitations. The cells are joined to one another with electron dense zonulae adherentes (za). The follicle cell basal lamina is next to the jelly space, and the ovarian lumen (ol) is on the opposite side. Scale bar = 0.25 μm. 39,200×.

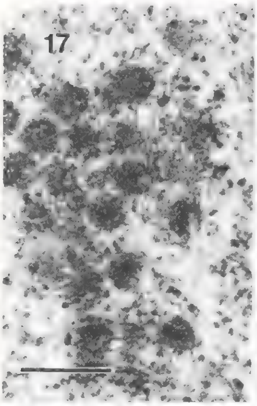
FIGURE 12. TEM of the oocyte basal lamina, the oolamina (oo). This separates the oocyte (O) surface from the hemal sinus (hs) of the connective tissue compartment. Scale bar = 2 μm 5,700×.

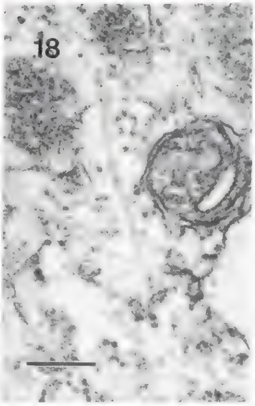
FIGURE 13. LM of the oocyte protuberance (pr) in an oocyte attached to the follicle (fe). The striations within the protuberance appear to exclude yolk granules from areas at the apex of the oocyte. The large germinal vesicle nucleus (gv) is eccentric towards this apex. Scale bar = 10 μm. 1000×.

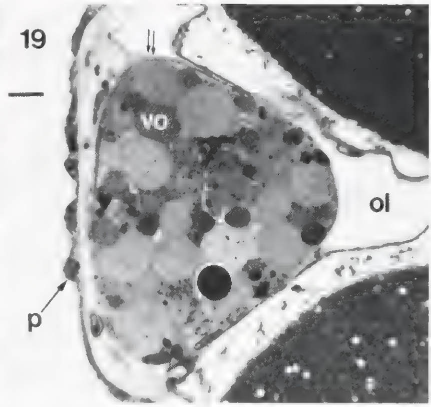
FIGURE 14. TEM of the protuberance in a grazing section showing the enormous number of microtubules (arrows) within the protuberance. The follicle cells (fe) are close by, and delimit the jelly space (js) surrounding the oocyte. Scale bar = 0.5 μm. 24,200×.

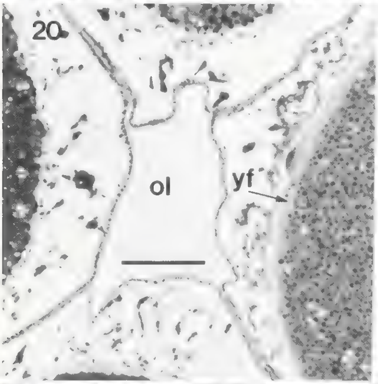
FIGURE 15. TEM of the insertion of the protuberance (pr) into the follicle cells (fe). The ovarian lumen (ol) and oocyte jelly space (js) are indicated. There are aggregations of vesicles within the follicle cells near the insertion of the protuberance (small arrow). Double arrows point out microtubules within the central region of the protuberance. The dense line between the protuberance and the follicle cells is the zonula adherens joining these cells. Scale bar = 1.0 μm. 8,200×.

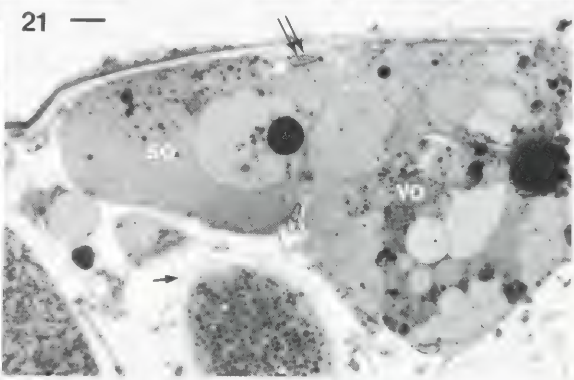
FIGURE 16. TEM of the oocyte cortex. Dense plaques occur at the plasma membrane (single arrows) and within vesicles near the oocyte surface. Microtubules underlie the oocyte surface and are indicated by double arrows. A yolk granule (yg) near the oocyte surface contains a crystal-like regular array of filaments. Scale bar = 0.5 μm. 25,500×.

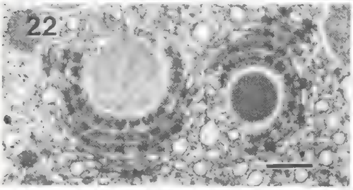


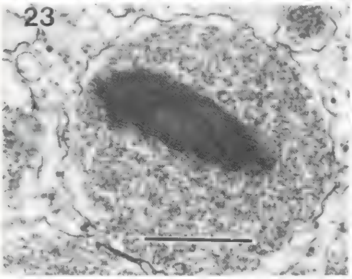












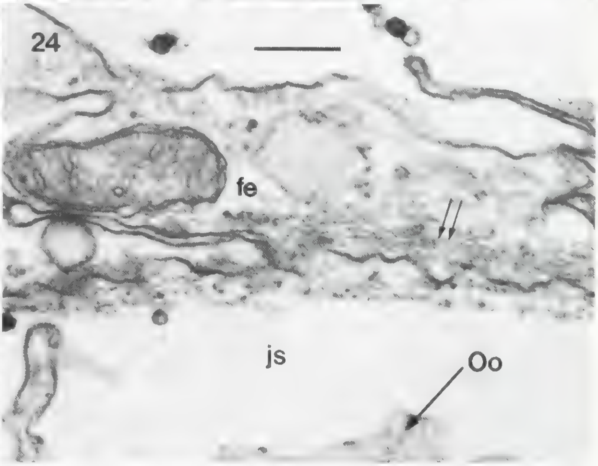


FIGURE 17. TEM of a grazing section of the nuclear envelope showing the nuclear pores and a microtubule. Microtubules appear to encircle the germinal vesicle. Scale bar = 0.25 μm. 48,000×.

FIGURE 18. TEM of a radially directed deep cytoplasmic mircotubule in the oocyte. The oocyte surface is about 20 μm beyond the top of the micrograph. Scale bar = 0.25 μm. 36,000×.

FIGURE 19. LM of a vesiculated oocyte (vo) in a fecund ovarian tubule. Vesiculated oocytes almost always occur at the periphery, near the peritoneum (p) of the ovary. They are separated from the ovarian lumen (ol) by cells of the inner epithelium. Double arrows indicate follicle cells. Scale bar = 10 μm. 420×.

Spawned tubules are resorbed within a few weeks. Resorption consists of further condensation in the length of the tubules and is accompanied by a visible deepening in their color. Sections through a resorbing tubule show the interior to be filled with partially degraded stunted oocytes and other cellular debris (data not shown). Marked cytolysis and phagocytosis of relict oocytes and other unidentifiable cells occurs. No follicle cells are found although there are numerous oolaminae visible. Secondary lysosomes and residual bodies abound in the perivisceral peritoneum of the resorbing tubules. Phagocytes containing dense aggregations of secondary lysosomes occur in the connective tissue compartment of these tubules and in some sections numerous morula cells also occur. Within several weeks, the only remnants of the fecund tubules are accumulations of dense pigment on the posterior of the gonad basis.

Discussion

The ovary of *Stichopus californicus* and all other holothurians is an azygous organ located in the anterior dorsal coelom. In most, but not all holothurians, the gonad is bilaterally symmetic about the dorsal suspensor mesentery. The gonopore is located in interambulacrum CD. Among the other eleutherozoan echinoderms, the gonads are multiple, and each has interambulacral gonopores. The unit of structure of holothurian ovaries is the ovarian tubule. These are naked, not surrounded by an outer sac of tissue (Ludwig, 1889; Gerould, 1896; Theel, 1901; Ohshima, 1921; Inaba, 1930; Hyman, 1955; Menker, 1980; Tyler and Gage, 1983). In other eleutherozoan gonads, ascini are the units of structure and an outer sac is present (Davis, 1971; Atwood, 1973b; Schoenmakers *et al.*, 1981; Walker, 1982; Buckland-Nicks *et al.*, 1984). These differences in echinoderm ovarian structure may be resolved by comparing holothurian ovarian tubules not to the adult gonad of other eleutherozoan echinoderms, but to the genital rachis, which has a shape and histological composition similar to the holothurian gonad (Cuenot, 1948; Hyman, 1955).

Peritoneum

In addition to serving as a protective layer for the ovary and containing the genital hemal sinus, the ovarian peritoneal epithelial cells probably absorb nutrients from the coelomic fluid. These cells bear numerous microvilli other than those which surround the single apical cilium. The fine structure is similar to the gonadal peritoneal epithelial cells of other holothurians (Atwood, 1973b; Davis, 1971). Krishnan and Dale (1975) suggest that the peritoneum of the testicular tubules of *Cucumaria frondosa* is capable of absorbing nutrients from the coelomic fluid. Nutrient absorption by peritoneal cells

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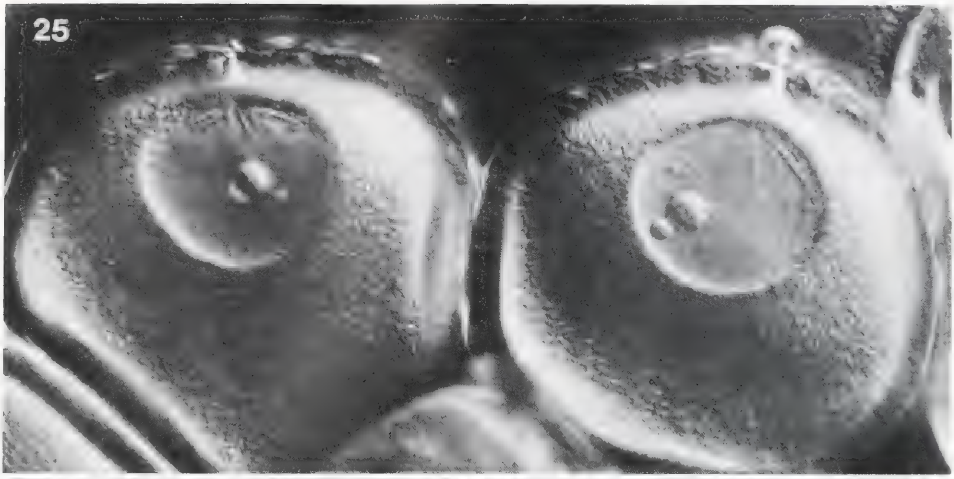
FIGURE 20. LM showing the ovarian lumen (ol) in a fecund ovarian tubule. The ovarian lumen is reduced to these interstices between follicle bound oocytes within the ovary, and the oocyte is separated from its neighbor by two layers of follicle cells. The oocyte on the right has a yolk free zone (yf) at its cortex. Scale bar = 10 μm. 1100×.

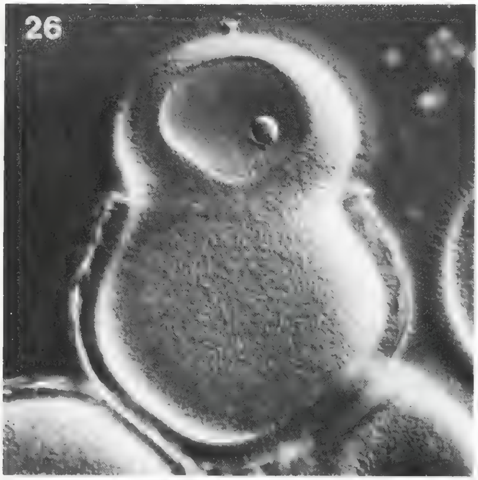
FIGURE 21. LM of a stunted oocyte (so) at the periphery of a fecund tubule. Like vesiculated oocytes (vo), stunted oocytes are rarely found in the interior of the tubule, and are surrounded by inner epithelial cells (double arrow). A complex pattern of microvilli is visible on the surface of a normal post-vitellogenic oocyte cut in grazing section (single arrow). Scale bar = 10 μm. 450×.

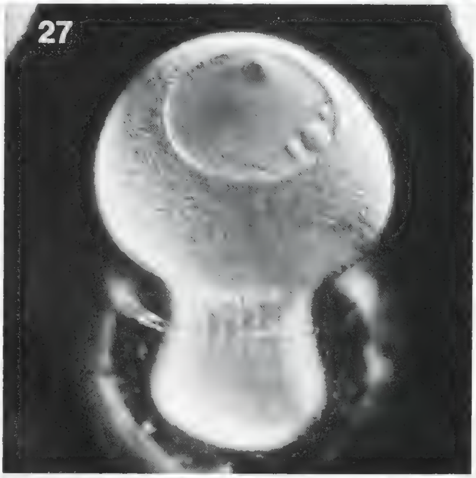
FIGURE 22. TEM of unusual annulate lamellae within the oocytes. Scale bar = 0.5 μm. 12,500×.

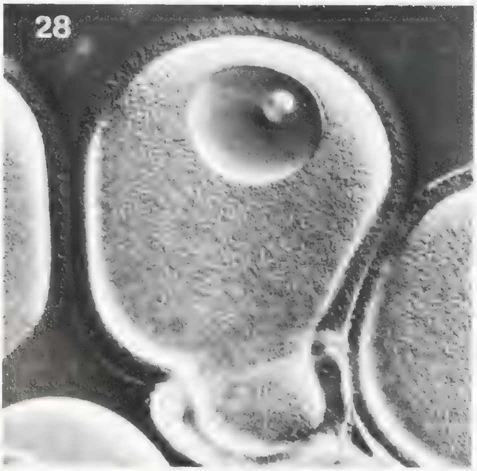
FIGURE 23. TEM of a yolk vesicle within the oocyte. A crystalline like structure is present within the yolk. Scale bar = 0.5 μm. 29,100×.

FIGURE 24. TEM of follicle cell (fe) and oocyte surface (Oo) during ovulation. The jelly space (js) is indicated. Five filaments within the follicle cells are indicated by the double arrows. Scale bar = 0.25 μm. 46,000×.









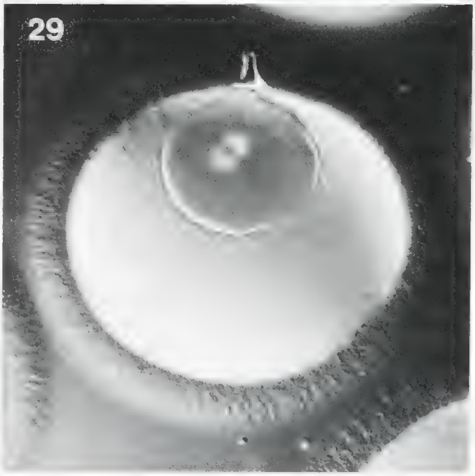


FIGURE 25. DIC micrograph of the first indication of ovulation. The protuberance (pr) of the oocyte on the left is still attached to its follicle cells. The protuberance of the oocyte on the right has poked through a small annulus in the follicle. 290×.

TABLE I

Summary of results of operative experiments on ovulating oocytes in which each oocyte follicle was torn with fine glass needles

Total	Stalled	Ovulated	Dead
50	4	39	7

along the length of the tubule coupled with the transfer of these nutrients to the genital hemal sinus could augment nutrient loads in the hemal fluid during vitellogenesis.

Nerves. Nerve processes are abundant in the peritoneum of the fecund ovarian tubules of *Stichopus californicus;* we estimate that as many as 24,000 axons may be present in each tubule. While the density of nerve processes is greater in the peritoneum of the *Stichopus californicus* ovarian tubules than has been reported for other eleutherozoan ovaries, it is difficult to estimate the total number of axons in those ovaries due to their complex shapes. This difficulty holds for perivisceral and hydrocoelic peritonea of other holothurians and other echinoderms (Atwood, 1973b; Baccetti and Rosati, 1968; Davis, 1971; Doyle, 1967; Jensen, 1975; Herreid *et al.*, 1976; 1977; Wood and Cavey, 1981). However, axons are plentiful in all examined echinoderm peritonea.

One hypothesis to explain the large number of axons in this ovarian peritoneum is derived by comparing the number of axons and muscle cells within a tubule. The correspondence of these numbers suggests that the mucle cells may be independently innervated. In all published reports of the fine structure of echinoderm perivisceral or hydrocoelic peritonea where dense assemblages of peritoneal myoepithelial cells are present, there are dense aggregations of axons. The possibility that muscle cells are independently innervated in this peritoneum is consistent with the absence of gap junctions between muscle fibers in this and a number of other echinoderm species (Prosser and Mackie, 1980; Cavey and Wood, 1981).

The aggregations of large, moderately electron dense vesicles (Fig. 5) are reminiscent of neurosecretory organs found in other animals (Baskin, 1976). Similarities in the size of the vesicles and their location suggests that they could have an endocrine function in this organ. The possibility that nerves within the echinoderm ovary could control oocyte maturation, ovulation, and spawning was suggested earlier for asteroids (Brusle, 1969; Holland, 1971; Atwood, 1973a). A potent neuroendocrine peptide is found in the radial nerves of a number of asteroids (Chaet and McConnaughy, 1959; Kanatani, 1979). Also, an aqueous solution of the radial nerve of the asteroid *Pycnopodia helianthoides* induces an increase in the percentage of oocytes of *Stichopus californicus* that undergo germinal vesicle breakdown (Strathmann and Satoh, 1969; Hufty and Schroeder, 1973). It is conceivable, therefore, that some endocrine factor may reside in the peritoneal neurons of this holothurian ovary.

FIGURE 26. DIC micrograph of the hour-glass stage of ovulation. The follicle lies about half way down the oocyte which is deformed at this point. 200×.

FIGURE 27. DIC micrograph of a late stage in ovulation. The follicle is particularly evident in this micrograph. The oocyte protuberance lies just out of focus over the germinal vesicle. 240×.

FIGURE 28. DIC micrograph of the final stage in ovulation. The oocyte has become nearly free of the follicle. This oocyte is slightly compressed. 180×.

FIGURE 29. DIC micrograph of an ovulated oocyte. The jelly coat surrounds the oocyte and the protuberance is topmost. 215×.

TABLE II

Summary of results from experiments where torn tubules were placed in a solution of cytochalasin B. The numbers are an average of the number of oocytes stalled in ovulation after 30 minutes of exposure to 1% DMSO in filtered seawater (Control), or 10 μg/ml cytochalasin B in 1% DMSO filtered seawater (Experimental)

Six replicate experiments; different females.	
Control	Experimental
2.17	47.5

Peritoneal musculature. The ovary of holothurians has been described as consisting of four layers, the peritoneum, a muscle layer, the connective tissue layer and the germinal epithelium (Ludwig, 1889; Gerould, 1896; Ohshima, 1921; Inaba, 1930). While the same layers appear in light micrographs presented in this study, detailed examination of the muscles in the *Stichopus californicus* ovary reveals that the muscle cells rest on the basal lamina of the peritoneum and have no external lamina. These facts support the interpretation that the muscle cells of this tissue are myoepithelial, and that the fecund ovarian tubule consists of only three principle layers. The same relationship of muscle cells to the peritoneal basal lamina has been found in other fine structural studies of perivisceral and hydrocoelic peritoneal epithelia in other holothurians (Atwood, 1973a; Baccetti and Rosati, 1968; Davis, 1971; Doyle, 1967; Doyle and McNiell, 1964; Herreid et al., 1976, 1977; Jensen, 1975; Pladellorens and Subirana, 1975). In the hydrocoelic or perivisceral peritonea of other echinoderms the musculature is superjacent to the peritoneal basal lamina and is interpreted as myoepithelial (Kawaguti, 1964, 1965; Cavey and Wood, 1981; Wood and Cavey, 1981). Further work is needed to determine if the peritoneal musculature of echinoderms is derived from the epithelial cells of the peritoneum or from mesenchyme.

In the gonads of some holothurians, longitudinal as well as circular muscles are present (Hyman, 1955; Davis, 1971; Atwood, 1973b; Franklin, 1980), while in others, only circular muscles are found (Gerould, 1896). The muscle layer of the fecund tubules of *Stichopus californicus* has a circular but no longitudinal component. A coordinated contraction of these circular muscles beginning at the distal end of the tubule and proceeding proximally is probably required to completely evauate the tubules during spawning. Those holothurians that release only a portion of their gametes at any single spawn during a reproductive season may have more elaborate control over their ovarian musculature. *Stichopus californicus* releases its eggs in a slow steady stream and not with the propulsive force reported for some tropical holothurians (Mosher, 1982). The differences in the force of gamete expulsion probably reflect differences in the organization of the tubule musculature as well as adaptations to particular ecological conditions.

In addition to its function in spawning, the peritoneal musculature of the tubule functions in peristalsis, which is visible in the tubules when the body cavity is opened. We suggest that peristalsis could mix nutrient rich hemal fluid at the periphery of the tubule with nutrient depleted hemal fluid deep in the longitudinal folds. Peristaltic contractions are more local and weaker than induced spawning contractions. These differences may reflect contractions of different sets of muscle cells or they may reflect the use of distinct neurotransmitters for each event. The presence of two kinds of vesicles within the peritoneal neurons, clear core and dense core, is compatible with both these suggestions.

Connective tissue compartment

The genital hemal sinus is the most obvious component of the ovarian connective tissue compartment in *Stichopus.* The sinus is a channel without cellular boundaries, with an organization similar to that of other echinoderms (Ruppert and Carle, 1984). A possible exception is the hemal 'vessel' of the holothurian *Cucumaria frondosa* (Doyle and McNiell, 1964) which is reported to bear an incomplete cellular inner lining or endothelium of fibroblast-like cells.

The histology of the genital hemal sinus within the ovary lends credence to the supposition that it supplies nutrients to the developing oocytes (Ludwig, 1889; Hyman, 1955; Walker, 1982; Ferguson, 1984). The role of the genital hemal sinus in providing these nutrients would be clarified if two questions were answered. First, how is longitudinal translocation of the nutrients along the length of the tubule possible? Second, how is radial translocation or replenishment of nutrient concentrations deep within the ovary accomplished?

On the basis of the hydrodynamic problems involved, it is difficult to support the contention that the longitudinal translocation of hemal fluid from the dorsal hemal sinus to the tips of the fecund tubules is the major route of nutrient transfer in the ovary. However, if nutrients are taken up by the peritoneum and passed to the hemal sinus all along the length of the tubule, it is possible to explain the radial translocation of the nutrient load toward the more centrally located oocytes by peristaltic contraction of the peritoneal musculature.

Inner epithelium

The inner epithelium of the *Stichopus californicus* ovary is continuous throughout the tubules, the gonad basis, and the gonoduct. The inner epithelium of fecund tubules consists of somatic and germ cells. The somatic cells are squamous for the greater part of the tubule's length, but close to the point where the tubule inserts into the gonad basis, the somatic cells become more cuboidal and the germ cells are absent.

Our description of the inner epithelium of the ovary supports previous observations on other holothurians (Ludwig, 1889; Gerould, 1896; Ohshima, 1921; Inaba, 1930). We have demonstrated that the oocytes are held within their follicles by intercellular junctions until the time of ovulation. While it is not certain that a cellular follicle surrounds all echinoderm oocytes, cellular follicles surround oocytes of ophiuroids (Patent, 1968) and asteroids (Schroeder *et al.*, 1979; Schoenmakers *et al.*, 1981). Strands of tissue, that may be cellular, surrounding echinoid oocytes have been depicted in drawings (Tennent and Ito, 1941) and micrographs (Pearse, 1970). In crinoids, oocytes are reflected into a basal lamina-lined cavity in the genital hemal sinus, but oocytes ovulate through a somatic inner epithelium (Holland, 1971; Holland and Dan, 1975). Although the primary function of somatic inner epithelial cells in all echinoderms is probably protection and the creation of a physiologically controllable microenvironment around the oocyte, some asteroid follicles act to produce the oocyte maturation hormone 1-methyladenine (Hirai and Kanatani, 1971). No endocrine function has been specifically localized in holothurian follicle cells.

Oocyte

Protuberance. The holothurian oocyte protuberance has been called the "micropyle appendage" to indicate homology between this structure and the jelly canal (micropyle) of the sea urchin ovum (Gerould, 1896; Ohshima, 1921). This term is confusing because it suggests that the oocyte is fertilizable at only one point on its surface. This suggestion is contradicted by our observations and is not corroborated in primary sources. It is likely that the recent report that sperm entry can occur at only one point on the holothurian oocyte surface is based on a misinterpretation of this term (Nieuwkoop and Sutasurya, 1983).

The oocyte protuberance has several functions; first it is the site of attachment of the oocyte to the follicle cells, second, its appearance is temporally associated with the migration of the germinal vesicle to its eccentric position in this holothurian, and third, it is the site of polar body formation, and marks the animal pole of the oocyte (Maruyama, 1980; Smiley, 1984).

Asteroid oocytes appear to be held within their follicle by basally directed processes from the follicle cells that make stable contacts with the oocyte surface (Schroeder *et al.*, 1979; Schroeder, 1981). It is likely that these processes serve the same attachment function in asteroids as the protuberance does in *Stichopus.* Information on how oocytes are attached to the inner epithelium is incomplete for both ophiuroids and echinoids. In the crinoid *Comanthus japonica,* the oocyte appears to have a structure somewhat similar to the holothurian protuberance (Fig. 11, Holland *et al.*, 1975).

The cytoplasmic striations associated with the holothurian oocyte protuberance have been reported to be homologous with a structure in asteroid oocytes called the polar plate (Buchner, 1911; Lindahl, 1932). The asteroid polar plate as well as similar axial structures found in oocytes of echinoids (Monne, 1946) and crinoids (Holland *et al.*, 1975) may serve as a center of aggregation of microtubules as does the protuberance of *Stichopus californicus.* This hypothesis is supported by the discovery of an ordered aggregation of microtubules between the germinal vesicle and the presumptive animal pole in the oocytes of the asteroid *Pisaster ochroceus* (Otto and Schroeder, 1984), although these investigators refer to this array of microtubules as a "premeiotic aster". The homology of this structure with the holothurian oocyte protuberance is supported on the basis of its location as well as its microtubular composition. The microtubules of the *Stichopus californicus* protuberance can be distinguished from the cortical microtubules in *Stichopus* and in the asteroid *Pisaster ochraceus* (Otto and Schroeder, 1984; Schroeder and Otto, 1984), by their axial location, their orientation, and their association with the nuclear envelope of the germinal vescile.

The protuberance develops late during oogenesis in *Stichopus,* when the oocyte has reached a diameter greater than 150 μm (Smiley, 1984). Late development of the protuberance also occurs in several other holothurians (Ohshima, 1921; Inaba, 1930), but in *Caudina arenata,* it is present in very small oocytes (Gerould, 1896). In all holothurian oocytes described, the germinal vesicle is centrally located until the time the protuberance becomes visible. Movement of the germinal vesicle to an eccentric position during vitellogenesis has also been reported in the holothurians *Cucumaria echinata* and *Caudina chilensis* (Ohshima, 1921; Inaba, 1930) and in the asteroid *Leptasterias hexactis* (Chia, 1968). The association of microtubules with the germinal vesicle in *Stichopus,* and the temporal concurrence of the development of the protuberance and the establishment of germinal vesicle eccentricity, suggests that the microtubules found within the protuberance may be involved in the movement of the germinal vesicle to its eccentric position, or its anchorage there. Otto and Schroeder (1984) suggested a similar function for the homologous microtubular aggregations in *Pisaster ochraceus.* Because germinal vesicle migration occurs at about the time of germinal vesicle breakdown in oocytes of *Holothuria leucospilota* and *Holothuria pardalis,* which are treated with dithiothreitol to induce oocyte maturation (Maruyama, 1980), the control of germinal vesicle migration may not be universal among holothurians.

The importance of three separable classes of microtubules within the *Stichopus* oocyte is not resolved. We have interpreted the microtubules of the protuberance as distinct from those of the cortex, as do Otto and Schroeder (1984) for the asteroid *Pisaster ochraceus.* On the basis of their differential stability in fixation, their distinct location, and different orientation, we also interpret the deep cytoplasmic microtubules found in *Stichopus* oocytes as a separate class of microtubules. This class of deep cytoplasmic microtubules has not, to our knowledge, been reported for any other oocyte. The difficulty in preserving these microtubules suggests that they may not have been preserved in other oocytes, although they could be present in the living cell.

The holothurian oocyte protuberance is a much more visible marker of oocyte polarity than the pigment band of *Paracentrotus lividus* oocytes (Boveri, 1901). The fact that the protuberance marks the site of polar body formation indicates the potential usefulness of this polarized axial marker in experimental manipulations of oocytes directed toward determining the cytological and biochemical properties of oocyte polarization.

Oolamina

Oocytes abut the hemal sinus at the oolamina, or oocyte basal plate. Because of its size and metachromatic staining, the oolamina is a convenient marker for the vegetal pole of the oocyte and for following changes in the ovary after ovulation. Nutrients in the hemal sinus must cross this barrier to enter the jelly space and contact the oocyte surface. In addition to acting as a sieve for the materials in the genital hemal sinus fluid, the oolamina may restrict passage of materials out of the jelly space, such as the maturation hormone during the hormone dependent period (Kanatani, 1979). Our ovulation studies also show that the oolamina can insure the integrity of the follicle if the stalk is severed from its connection with the parietal inner epithelium.

Ovulation

The junctions between oocytes and somatic inner epithelial cells in echinoderm ovaries must be ruptured before oocytes can be released into the sea. This may explain why asteroids and holothurians do not release oocytes when injected with isotonic potassium chloride solutions (Hyman, 1955). Ovulation has been described for a crinoid (Holland and Dan, 1975) and several asteroids (Schroeder, 1971). Crinoid ovulation involves distortion of the oocyte into an hourglass shape but contraction of the somatic cells of the inner epithelium has not been reported. In asteroid ovulation, follicle cells contract more independently and oocyte distortion is minimal. During ovulation in *Stichopus californicus,* the oolamina and follicle cells remain attached to the inner epithelium of the ovary, and ovulated oocytes accumulate in the ovarian lumen. With the collapse of the oocyte follicles, the ovarian lumen encompasses the greater part of the volume of the ovary. Continuous and asynchronous peristaltic contractions of the peritoneal musculature begin at the distal end of the ovarian tubule and move the oocytes within the lumen. Later, oocytes will spill from the gonoduct of the animal. The jelly coats of spawned oocytes are intact; in *Stichopus,* oocytes are not spawned with their follicles, though the release of oocytes in their follicles has been reported for *Cucumaria elongata* (Chia and Buchanan, 1968).

The experimental analysis of the process of ovulation does not prove that contraction of actin filaments produces all the forces involved in ovulation, but it does indicate that actin filament contractions are involved. It is unfortunate that the oocytes of *Stichopus californicus* are intensely autofluorescent at the wavelength of the fluorophores fluorescein isothiocyanate and NDB. When different fluorophores are available, it would be interesting to study the involvement of microfilaments in the oocytes as well as in the follicle cells to determine the relative contribution of each of the process of ovulation.

Resorption of spawned tubules

Stichopus californicus completely resorbs the spawned fecund tubules in the weeks immediately following spawning. The process of resorption in *Stichopus* is very similar to that reported for *Mesothuria intestinalis* (Theel, 1901) and *Ypsilothuria talismani* (Tyler and Gage, 1983). Post spawning resorption of spent tubules was reported for other holothurians by Hyman (1955) who suggested that it would prove to be the rule among holothurians. Investigations on the reproductive biology of several holothurians do not indicate that resorption has occurred after spawning (Tanaka, 1958; Conand, 1981). The complete resorption of the fecund tubules in *Stichopus* obscures the source of future oocytes, unless they come from the smaller tubules more anterior on the gonad basis.

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HATCHING OF *ILYANASSA OBSOLETA* EMBRYOS: DEGRADATION OF THE EGG CAPSULE PLUG IN THE ABSENCE OF DETECTABLE PROTEOLYSIS OF THE MAJOR PLUG PROTEINS

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ABSTRACT

The mechanism of action of the hatching substance released by *Ilyanassa obsoleta* embryos was examined by studying the sequential ultrastructural and biochemical changes that occur in the egg capsule plug as it is dissolved during hatching. When release of the hatching substance is triggered by incubating prehatching embryos in KCl, the inner two layers of the capsule wall (L3 and L4) that extend into the apex to form the plug separate from one another, but only in this region. The hatching substance then dissolves material at the periphery of the plug so that an intact plug can be recovered. However, if the plug is left in contact with the hatching substance, both the thin, electron dense material of the inner layer (L4) and the 10 nm filaments of the adjacent layer (L3) that compose most of the plug are dissolved. The first step in the hatching sequence is mimicked by papain so that L3 and L4 can be separated for analysis on SDS-polyacrylamide gels. L3 contains four major proteins with molecular weights of 24,000–52,000 daltons while L4 contains a predominant 25,000 dalton protein. When isolated plugs are dissolved in crude preparations of the hatching substance and analyzed by polyacrylamide gel electrophoresis, there is evidence of only slight disappearance of one minor plug protein. Based on these findings, *Ilyanassa* embryos probably release several activities necessary to dissolve the plug, yet degradation of the plug occurs without hydrolysis of the major plug proteins.

INTRODUCTION

Extracellular envelopes, which surround the eggs and embryos of nearly all animals, pose a barrier that must be penetrated at two critical periods of development. At fertilization, sperm must digest a passageway through these envelopes to successfully fuse with the egg cell surface. Accordingly, spermatozoa of many animals contain enzymes (McRorie and Williams, 1974; Hoshi *et al.,* 1979; Green and Summers, 1982; Yamada and Aketa, 1982) or lysins (Haino-Fukushima, 1974; Lewis *et al.,* 1982; Ogawa and Haino-Fukushima, 1984) within the acrosome that are released during fertilization. Development continues until the time of hatching when the envelopes must be penetrated again, this time by the embryo. Embryos escape from an envelope by one (or a combination) of three methods: an osmotic influx of water into an envelope to rupture it; mechanical movements of the embryo to break it; or the release of specific chemicals to weaken or dissolve it (reviewed by Davis, 1968). In most systems examined thus far, the chemicals released by embryos at hatching and those released by sperm during fertilization appear to be very similar to one another.

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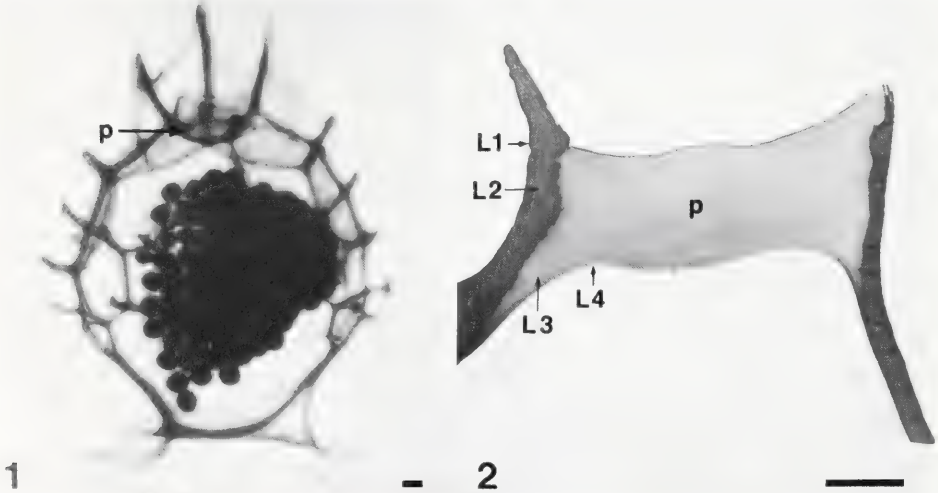
In these systems, sperm and embryos release divalent cation-requiring proteases, which is not surprising since envelopes are composed primarily of proteins (Katagiri, 1975; Bleil and Wassarman, 1980; Schoots and Denuce, 1981; Urch and Hedrick, 1981a).

The emergence of sea urchin and amphibian embryos from the fertilization envelope (a primary envelope produced by the oocyte) and fish embryos from a chorion (a secondary envelope derived from follicle cells) are the only systems in which the hatching process has been studied in detail. Considerable information is available on the hatching enzymes released by these embryos (see Sullivan and Bonar, 1984) yet the mechanism of action of these enzymes has been studied only during fish (Yamamoto and Yamagami, 1975; Iuchi and Yamagami, 1976; Yamagami, 1981) and frog (Yoshizaki, 1978; Urch and Hedrick, 1981b) hatching. Furthermore, similar data are not yet available on hatching from tertiary envelopes (secreted along the oviduct), so that it is impossible to draw any conclusions on the ubiquity of the hatching process for diverse animal groups.

One example of an oviduct-derived tertiary envelope is the multi-layered gastropod egg capsule. Of the few species of snails for which chemical hatching has been described, *Ilyanassa obsoleta* was chosen for our studies because adults reproduce readily and females deposit an abundance of egg capsules (Fig. 1) in the laboratory and because hatching is an early developmental event (Scheltema, 1967). After about a week of development embryos hatch as swimming veligers by releasing a hatching substance that dissolves the capsule plug, which occupies a small region of the egg capsule apex (Pechenik, 1975).

We have shown that the *Ilyanassa* hatching substance is a divalent cation-requiring protein that shares many of the biochemical characteristics of other well studied hatching enzymes (Sullivan and Bonar, 1984). It remains to be determined if the *Ilyanassa* hatching substance is also a protease. Along with biochemical studies on the hatching



FIGURES 1 AND 2. Light micrographs of the *Ilyanassa obsoleta* egg capsule. The plug (p) is at the apex of the capsule (Fig. 1). The location of the four layers of the egg capsule wall is revealed from a cross section through the apex (Fig. 2). The outer layer (L1) and the second layer (L2) form most of the capsule wall while the third layer (L3) expands in the apex to form most of the plug (p). The inner layer (L4) is not resolved by light microscopy, but its position lining the embryonic chamber is shown. Bar = 100 μm.

substance itself, we have undertaken an alternative approach to studying the function of this protein by examining its action on its natural substrate, the egg capsule.

In this study, we have identified three steps to the hatching process: (1) separation of the innermost capsule wall layer (L4) from its adjacent layer (L3) only in the plug region; (2) digestion of possible "cementing" material around the periphery of the plug; and (3) plug degradation itself. The first step of the hatching sequence can be mimicked by the protease papain, suggesting that the hatching substance has protease activity. However, the primary activity of the hatching substance, that of dissolving the plug, occurs in the absence of proteolysis of the major plug proteins, so that it is not clear whether the hatching substance is in fact a hatching enzyme.

MATERIALS AND METHODS

Development of embryos

Ilyanassa adults, obtained from the Marine Biological Laboratory in Woods Hole, Massachusetts, or collected from mudflats near Lewes, Delaware, were maintained in aerated artificial seawater (Instant Ocean), 28‰ at 10°C. Groups of 35–40 snails were transferred to a 5 gallon aquarium at 23°C and fed frozen shrimp every other day to initiate reproduction. Egg capsule deposition began within a few days and capsules were collected daily from the aquarium walls. Capsules were rinsed briefly in 50% ethanol and cultured at 23°C in filtered seawater containing 20 μg/ml of the antibiotic rifampicin. Under these conditions, the majority of embryos hatch on day eight of development (Sullivan, 1984), yet it is difficult to predict when hatching from an individual capsule will occur. However, we have been able to experimentally initiate release of the hatching substance from embryos by briefly incubating embryos in a solution containing 280 m*M* KCl (60% 470 m*M* KCl and 40% K^+-free seawater, hereafter referred to as KCl) (Sullivan, 1983). With this technique, it has been possible to synchronize hatching from many capsules, required for the experiments outlined below.

Transmission electron microscopy

In order to observe the sequential changes in the capsule ultrastructure following release of the hatching substance, groups of 25 eight-day-old capsules were placed in a 10 ml dish of KCl to trigger release of the hatching substance. At five minute intervals, five capsules were removed and were immediately fixed in 5 m*M* 3-(N-Morpholino)propanesulfonic acid (MOPS) buffered seawater (pH 8.0) containing 2.5% glutaraldehyde. Capsules were cut in half below the plug and the apical region was postfixed in 1% aqueous OsO_4, dehydrated through an ethanol series, and embedded in Epon as described earlier (Sullivan and Maugel, 1984). Sections were cut with a diamond knife on a Sorvall MT-2B ultramicrotome, collected on copper grids, and stained with 2% aqueous uranyl acetate and 0.2% aqueous lead citrate (Venable and Coggeshall, 1965). Specimens were examined and photographed on a Hitachi HU-12 or an RCA EMU-3H transmission electron microscope.

Polarity of action of the hatching substance

Hatching enzymes normally digest inner portions of an envelope, then progressively digest outer layers. In sea urchin embryos, however, synchronous hatching occurs because the hatching enzyme released from older embryos can degrade the fertilization envelopes of younger embryos from the outside-in (Kopac, 1941). Therefore, we tested whether the *Ilyanassa* hatching substance could digest capsule plugs from the outside-in as well as from the inside-out by placing intact capsules or opened capsules in preparations of crude hatching seawater prepared as described previously (Sullivan and Bonar, 1984). Twenty 8-day-old capsules were cut open into a solution of seawater and capsule fragments were removed. The embryos were then gently pelleted and seawater was withdrawn and replaced with a solution of KCl to induce release of the hatching substance. After 45 minutes, embryos were pelleted again and the resulting supernatant served as a crude preparation of the hatching substance. The predominant embryonic protein in this solution is a 55,000 dalton protein which may be the hatching substance. Before an experiment a 50 μl aliquot of each batch of the hatching substance was tested for its biological activity of removing a plug from the apex of a newly deposited capsule.

Incubation of egg capsules with enzymes

We attempted to mimic the action(s) of the hatching substance by monitoring the sensitivity of the capsule and plug to enzymatic digestion. One-day-old capsules were opened and the embryos and capsule fluid washed away. Capsule fragments were incubated in various commercial (Sigma) proteases (chymotrypsin, elastase, papain, pepsin, pronase, proteinase K, trypsin) or carbohydrases (snail gut enzymes, hyaluronidase, chitinase) at 1 mg/ml in an appropriate buffer for up to six hours and monitored for obvious changes in morphology.

Polyacrylamide gel electrophoresis

When isolated capsule plugs are homogenized in sample buffer (Laemmli, 1970), and examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), three glycoproteins are resolved having molecular weights of 49,000, 29,000, and 24,000 daltons (Sullivan and Maugel, 1984). In the initial analysis of plugs by SDS-polyacrylamide gel electrophoresis proteins were identified with Coomassie blue and the periodic acid-Schiff's stains. In the experiments below, we used the highly sensitive silver stain procedure (Oakley *et al.*, 1980) which allowed us to use fewer plugs per experiment while still detecting all three plug proteins in our samples. In one set of experiments plugs dissected from newly deposited capsules or plugs released from day eight capsules following KCl treatment were examined for similarities in protein profile to confirm that the proteins we identified were all from the plug and not part of the capsule wall. It was also possible to examine the proteins present in both regions of the plug (L3 and L4) because these layers could be separated by enzyme treatment. A final group of experiments was designed to determine if any (or all) of the plug proteins were sequentially degraded as hatching proceeded by placing isolated plugs in crude preparations of the hatching substance prepared as described above. Groups of twenty intact plugs were added to a 50 μl aliquot of the crude hatching substance for various times and digestion was stopped by the addition of an equal volume of 20% trichloroacetic acid (TCA) to precipitate proteins. Plugs that were soft and partially digested (see Fig. 4) were collected after six hours and completely digested plugs were collected at 12 and 24 hours. A similar series of digestions were performed on isolated pieces of L4. TCA precipitable material was pelleted at 10,000 $\times g$ and the pellet was resuspended in 1$\times$ Laemmli sample buffer, heated at 95°C for 2 minutes, and loaded onto a 12.5% polyacrylamide gel. Molecular weight markers (Pharmacia) were separated on each gel for determination of the molecular weights of plug proteins (Weber and Osborn, 1969).

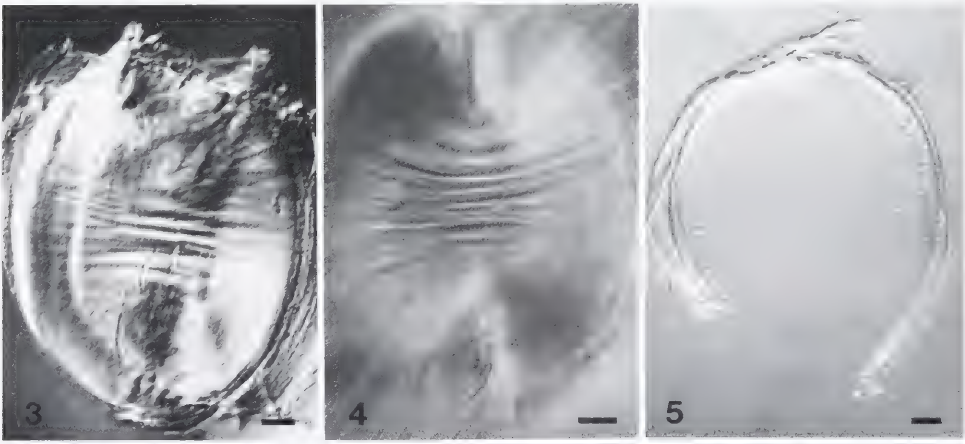
RESULTS

Changes in capsule morphology during hatching

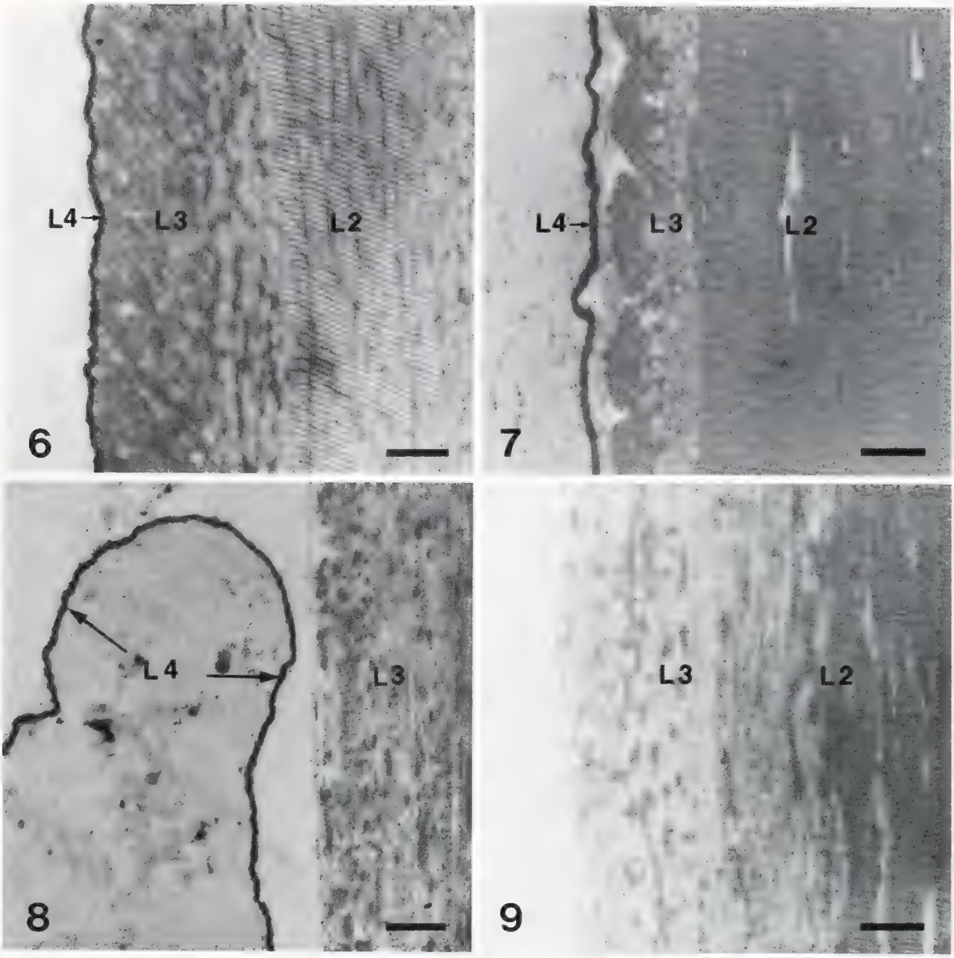
The *Ilyanassa* egg capsule wall contains four distinct layers that are most easily distinguished by their appearance in transmission electron micrographs (Sullivan and Maugel, 1984). The outer two layers of the capsule, L1 and L2, extend into the capsule apex and are adjacent to the plug but do not contribute material to the plug. The inner two layers of the wall, L3 and L4, are present throughout the capsule and extend into the apical region forming the plug, which is predominantly L3 (Fig. 2).

The capsule plug is a small disk 400–500 μm in diameter and 100–150 μm in thickness (Fig. 3). Plugs can be dissected manually from capsules, or will drop out intact from whole capsules within 10–15 minutes of KCl treatment. When plugs are exposed to the hatching substance for longer periods, they become soft and diffuse (Fig. 4). During natural hatching, plugs are quickly dissolved, but when isolated plugs are placed in crude preparations of the hatching substance, dissolution is slowed, probably because the hatching substance is less concentrated. Whereas a plug dissolves naturally in about an hour, isolated plugs take between 2–4 hours to dissolve in our experiments. Such plugs are completely dissolved, while portions of the adjacent capsule wall (probably L2) remain (Fig. 5).

When hatching is initiated by placing intact eight-day-old capusles in KCl, the various steps of the hatching process leading to plug removal can be observed. No changes in the capsule ultrastructure were noted within the first five minutes of KCl treatment. We believe the hatching subtance has been released, but has not had sufficient time to act. Capsule walls appeared indistinguishable from those of newly deposited capsules (Sullivan and Maugel, 1984). The striated fibers of L2, the filaments of L3, and the electron dense layer of L4 remained intact and closely apposed (Fig. 6). After 10 minutes, the hatching substance has caused L4 to separate from L3 (Figs. 7, 8). Although L4 appears uniform throughout the egg capsule (Sullivan and Maugel,



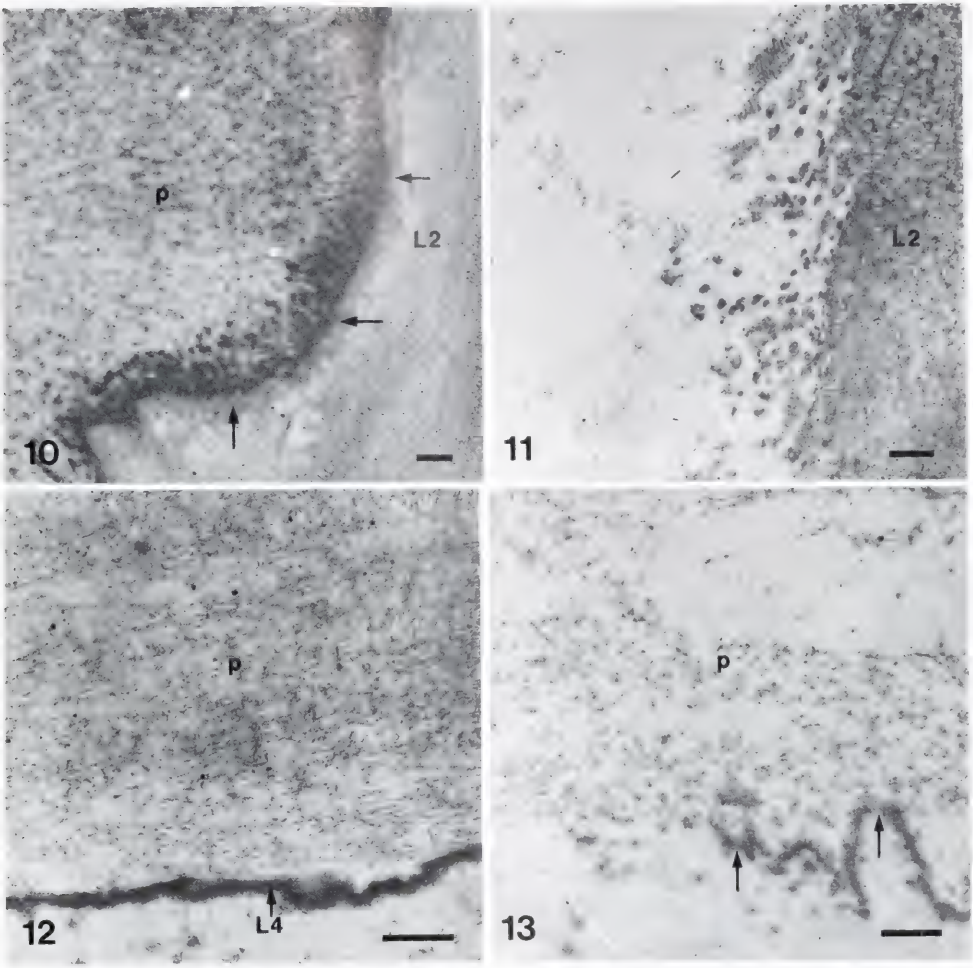
FIGURES 3–5. Nomarski interference contrast light micrographs of the digestion of an egg capsule plug. Prior to day eight, plugs remain firmly in place in the capsule apex but can be dissected from this area (Fig. 3). When a capsule plug is added to the hatching substance, plug filaments become very diffuse within 60 minutes (Fig. 4). With prolonged incubation in the hatching substance, the plug has dissolved, while material from the adjacent layer remains undigested (Fig. 5). Bar = 50 μm.



FIGURES 6–9. Transmission electron micrographs of *Ilyanassa* egg capsules incubated in KCl to initiate release of the hatching substance. After five minutes of KCl treatment, no changes are visible in the capsule wall (L2–L4 are shown with the inside of the capsule to the left) and all layers remain in close contact (Fig. 6). By 10 minutes L4 has begun to separate from L3 slightly below the apex (Fig. 7) but has pulled away considerably in the plug region (Fig. 8). After 15 minutes of treatment, the first phase of hatching is completed when L4 has been completely removed from the apex and the hatching substance has access to the filaments of L3 (Fig. 9). Bar = 0.5 μm.

1984), it separates from L3 and dissolves only in the capsule apex (Fig. 9). In other regions of the capsule, L4 remains tightly adherent to L3.

Around this time, L4 begins to dissolve and the hatching substance now has access to inner regions of the capsule apex. The next change in the capsule structure occurs at the margins of the plug where it contacts L2. The area of close contact between the plug and the fibers of L2 (Fig. 10) begins to loosen, perhaps as a result of the hatching substance digesting material holding the plug in place. The hatching substance apparently works very quickly because within 15 minutes of KCl treatment, material present between the plug and L2 (Fig. 10) is no longer present (Fig. 11) and intact plugs can be recovered from about 50% of the capsules tested (Sullivan, 1983).



FIGURES 10–13. Transmission electron micrographs of the second and third phases of the hatching process. During the second phase the area of close contact between L2 and the plug (p) is disrupted, possibly by the digestion of material between these layers (arrows) that may hold the plug in place (Figs. 10, 11). During the final step of the hatching process the two layers of the plug (L3 and L4) that remain intact during the first 15 minutes of KCl treatment (Fig. 12) are quickly digested by the hatching substance (Fig. 13). Bar = 0.4 μm.

If a capsule plug is left undisturbed, both the 10 nm filaments of L3 and the 60 nm wide electron dense material of the L4 are quickly dissolved (Figs. 12, 13), so that after an hour the plug is gone. Therefore, hatching is divided into three phases: separation of L4 from L3; degradation of material at the periphery of the plug so that a plug can be recovered intact; and dissolution of the plug itself.

Polarity of the hatching substance

When intact capsules were incubated in crude preparations of the hatching substance so that only the external plug surface was exposed, plugs were never removed.

Control capsule apices cut open to expose the inner side of the plug to the hatching substance had their plugs dissolved in all replicates of this experiment.

Protease and carbohydrase action on the capsule

Attempts to mimic the action of the hatching substance with commercial proteases or carbohydrases were unsuccessful with one exception. Incubation of capsule apices in papain (10 mg/ml in 10 m*M* Tris pH 7.6 diluted 1:1 with filtered seawater) loosened the contact between L4 and L3 so that L4 could be separated easily from the rest of the capsule (Fig. 14). With this 0.5% solution of papain, L4 began to separate from L3 in less than 60 seconds, with less concentrated papain solutions taking longer.

Polyacrylamide gel electrophoresis

Silver stained gels of *Ilyanassa* egg capsule plugs had protein profiles that differed somewhat from our earlier results (Sullivan and Maugel, 1984). The 49,000 dalton band was resolved into a doublet of 52,000 and 48,000 daltons and additional proteins with molecular weights in excess of 95,000 daltons were detected (Fig. 15a).

In our previous work, whole plugs were dissected from capsule apices. It was possible that some of the proteins resolved on gels were part of L2 adhering to the plug (see Fig. 5). Therefore, we treated capsules with KCl to separate plugs from the wall prior to analyzing plug proteins. It was also possible to determine whether any of the plug proteins identified previously were from L4 by removing this layer from the remainder of the plug with papain. With these additional two treatments, identical protein profiles were observed (Figs. 15b, c) indicating that the four proteins we have identified were in fact from the plug, and furthermore, all were from L3.

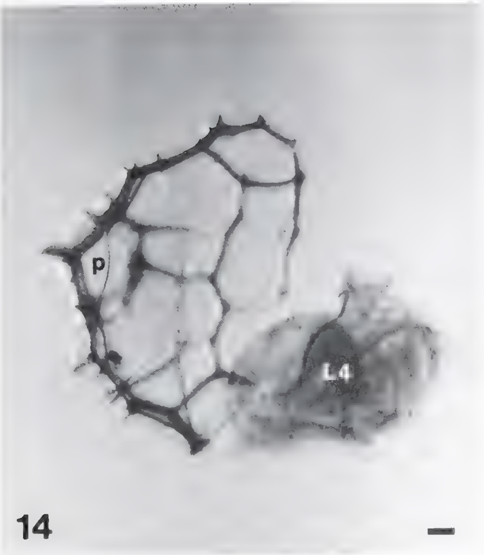
Fragments of L4 that were separated from L3 with papain were also analyzed by gel electrophoresis. L4 contained a prominent 25,000 dalton protein (Fig. 15d) and occasionally a faint 15,000 dalton protein was detected (see Fig. 17).

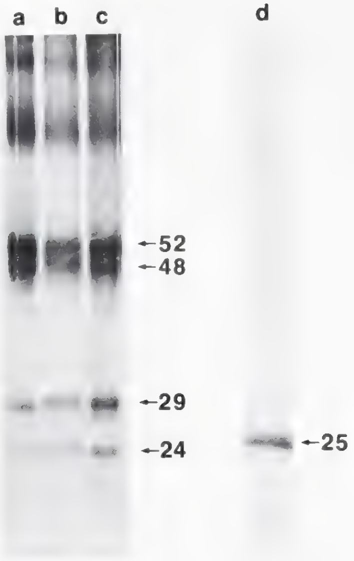
Plugs that have been in contact with the crude hatching substance for about six hours and are partially dissolved, (see Fig. 4) still contain the same four proteins seen in plugs from newly deposited capsules (Fig. 16). These four proteins persist intact for up to 24 hours even when digestion has gone to completion and plugs are no longer visible. We also analyzed samples of L4 for evidence of proteolysis by the hatching substance. There is only minor reduction of the 25,000 dalton protein by 24 hours, well after this layer has been digested by the hatching substance (Fig. 17).

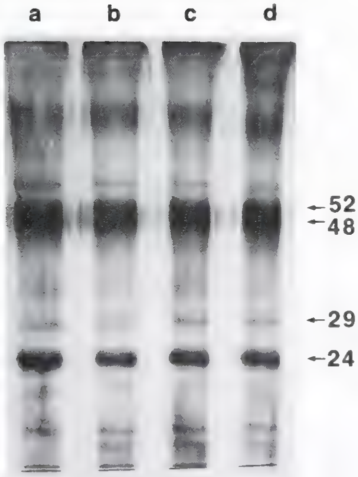
DISCUSSION

Although hatching from egg envelopes is a ubiquitous problem for embryos, what is known about chemical hatching has come from studies of very few species whose embryos emerge from primary or secondary envelopes. We have selected *Ilyanassa* as an example of embryos enclosed within a tertiary envelope to compare its hatching mechanism to what is known about the hatching processes and hatching enzymes of the other more thoroughly studied embryos.

Much of the early work on the characterization of hatching enzymes utilized artificial protein substrates which generally are not degraded very quickly, suggesting that the enzymes have high specificity for their natural substrates, which are degraded very rapidly (Ishida, 1944; Kaighn, 1964). The preferred approach now is to analyze the natural substrate of a hatching enzyme directly (Yamagami, 1970; 1973). For example, products released from radioactively labeled envelopes can be quantitated under a variety of conditions (pH, temperature, ions, protease inhibitors) to study the







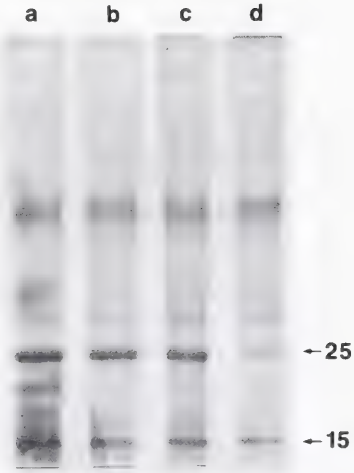


FIGURE 14. Light micrograph of a capsule apex treated with 0.5% papain. The innermost layer (L4) quickly separates from the rest of the capsule wall, but the plug (p) remains in place. Bar = 50 μm.

FIGURE 15. Analysis of *Ilyanassa* capsule plug proteins on a 12.5% polyacrylamide gel. Plugs were dissected from newly deposited capsules (a), were released from 8-day capsules following incubation in KCl for 20 minutes (b), or were incubated in papain to remove L4 then dissected from capsules (c). Pieces of L4 were also analyzed (d). Molecular weights (in kdaltons) of the major proteins are indicated.

FIGURES 16–17. Analysis of digestion of capsule plug proteins on a 12.5% polyacrylamide gel. Entire plugs (Fig. 16) or pieces of L4 alone (Fig. 17) were incubated in the hatching substance for 0, 6, 12, and 24 hours (lanes a–d) then prepared as described. Locations of the major proteins (in kdaltons) are indicated. The high background staining on these gels is due to proteins present in the crude hatching substance.

activity of a hatching enzyme (DiMichelle *et al.*, 1981; Urch and Hedrick, 1981a). Another useful approach is to examine sequential biochemical or ultrastructural changes in an envelope as a consequence of the release of a hatching enzyme (Yamamoto and Yamagami, 1975; Iuchi and Yamagami, 1976; Yoshizaki, 1978; Urch and Hedrick, 1981b). We have utilized these latter approaches in our analysis of the *Ilyanassa* hatching substance to determine how it functions and if it may be a protease.

Our results support an earlier conclusion that the onset of hatching is rapid and is controlled by the sudden release of the hatching substance (Sullivan, 1983). There is no evidence from transmission electron micrographs that any layer of the capsule wall or plug is altered prior to day eight, nor do the plug proteins undergo any gradual degradation during the prehatching period. Hatching is also a very sudden event for some fish embryos with the rapid digestion of the *Oryzias latipes* chorion (Yamamoto and Yamagami, 1975) being accompanied by the release of at least six soluble glycoproteins (Iuchi and Yamagami, 1976). However, two high molecular weight proteins of the vitelline envelope around *Rana japonica* embryos are slowly degraded during the two to three days preceeding hatching (Yoshizaki, 1978). Therefore, hatching is a gradual process for the embryos of this frog.

There are two other features of the hatching mechanism utilized by embryos that we have examined for *Ilyanassa*. As mentioned above, some hatching enzymes degrade an envelope equally well from either side. This is not the case for *Ilyanassa*. Although the hatching substance can degrade all parts of the egg capsule plug, it apparently must do it in the proper sequence. Another difference is that while the envelopes of fish, frog, and sea urchin embryos are entirely dissolved during hatching, almost all of the *Ilyanassa* egg capsule persists after hatching. It is not clear why L4, which lines the entire embryonic chamber (Sullivan and Maugel, 1984), is only digested in the capsule apex. Removal of L4 from the apex gives the hatching substance access to the L3 portion of the capsule plug leading to plug digestion, yet L3 also remains intact throughout the rest of the capsule. The outer two layers of the capsule wall also remain intact so that much of the capsule of *Ilyanassa*, like those of other marine gastropods, is resistant to hatching "enzymes" (Fretter, 1941). This finding is best explained by differences in amino acid composition of capsule plugs and the outer layers of the capsule wall. For example, amino acid analysis of the *Busycon* capsule walls failed to detect cysteine or tryosine residues, while both of these amino acids were detected in protein hydrolyzates of plugs (Harasewych, 1978), leading to the conclusion that the *Busycon* hatching enzyme must have chymotrypsin-like specificity. Similarly, histochemical tests for specific amino acids revealed that stains for tyrosine and tryptophan residues were strongly positive in the plug of the *Nucella* capsule while the capsule wall was deficient in these amino acids (Bayne, 1968).

We have implied that the hatching substance is a single functional protein with one activity, but this probably is not the case. Results from the ultrastructural analysis of hatching suggest that there could be three or four separate activities released by embryos that are needed to separate L4 from L3, to digest material at the plug-wall junction, and to dissolve L4 and L3. Therefore, it is not a simple matter to assign a mechanism to what may be a very heterogeneous hatching substance. Because of the protein composition of the capsule plug, it is tempting to speculate that the hatching substance would be a protease, but the plug and entire capsule are very resistant to digestion by almost all of the proteases tested. One of the early events of hatching is mimicked by papain, so perhaps the hatching substance has a proteolytic component, but the primary activity of dissolving the plug is occurring by very limited proteolysis of the plug. The four major proteins of L3 that compose virtually all of the plug have identical molecular weights on polyacrylamide gels when we analyze samples of plugs that are either intact, partially digested, or completely dissolved. Thus the major activity of the hatching substance is not as a protease that degrades these four proteins. It is possible that the major plug proteins are held together by lower molecular weight cross-linking proteins and it is these cross-linking proteins that are the substrate of the hatching substance. We have not detected any such proteins when plugs were examined on 20% polyacrylamide gels, suggesting that if such cross-linking proteins exist, they would have molecular weights less than 8–10,000 daltons.

An alternative explanation for the action of the hatching substance is that it is functioning as a lysin. The primary criterion for distinguishing a lysin from an enzyme is how it interacts with its substrate: either catalytically to release distinct products (an enzyme), or stoichiometrically, remaining bound and not releasing distinct products (a lysin) (Lewis *et al.*, 1982). Two observations suggest the hatching substance of *Ilyanassa* may be working in a manner consistent with it being a lysin. First, the four plug proteins from L3 were not degraded by the hatching substance when analyzed on SDS-polyacrylamide gels. Similarly, there is no change in the migration of the major vitelline envelope proteins before and after prolonged incubation with the *Haliotis* sperm lysin (Lewis *et al.*, 1982). Second, it appears that the plug dissolving activity of crude preparations of the hatching substance can be saturated when additional plugs are added. In our assays of hatching activity, a single plug is usually dissolved in two or three hours (Sullivan and Bonar, 1984). However, when additional plugs are added to separate aliquots of the same batch of the hatching substance, plug digestion no longer occurs (Sullivan, unpub.). A true enzyme would be expected to dissociate from its substrate and be available to work on additional plugs, but a lysin remains bound to its substrate.

We have detected low levels of protease activity released at hatching (Sullivan and Bonar, 1984) that may correspond to the papain-like activity that separates L4 from L3. Therefore, there may be a proteolytic component to the hatching substance. However, the primary activity of the hatching substance is not one that hydrolyzes the major plug proteins. These results provide valuable information on how we should approach the characterization of the hatching substance of *Ilyanassa* in our future experiments.

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THE INFLUENCE OF SEX AND TREMATODE PARASITES ON CARRION RESPONSE OF THE ESTUARINE SNAIL *ILYANASSA OBSOLETA*

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ABSTRACT

The estuarine neogastropod, *Ilyanassa obsoleta,* was studied to determine the influence of sex and trematode parasites on the well-known tendency of this snail to aggregate on carrion. Fifteen experimental arenas (1 × 5 m) were delimited on the Cape Henlopen sandflat in Delaware Bay between July 1982 and November 1983. Snails (n = 2111) were examined by dissection. Frequencies of snails in specific categories of sex and parasitism in the arenas were determined before carrion was made available (expected frequencies), and these frequencies were compared with frequencies of the same categories among snails responding to carrion (observed frequencies). Experiments were categorized for analyses into breeding and nonbreeding temporal groups based upon the presence/absence of females with egg cases. Sexual condition alone affected carrion response. Uninfected females in reproductive condition tended to respond more frequently than predicted by controls (positive response) both during and after reproductive season, or else responded as predicted (neutral response). During breeding season males tended to show a neutral response and afterward to respond less frequently than expected (negative response). Parasite influence on carrion response was not detected until after the breeding season, at which time it was pronounced. Both sexes showed a positive response when infected with *Lepocreadium setiferoides. Zoogonus rubellus* and *Gynaecotyla adunca* infections produced negative responses in females but neutral responses in males. *Austrobilharzia variglandis* was inhibitory to females and probably to males as well. *Himasthla quissetensis* infections seemed not to affect response of either sex. Multiple infections were relatively frequent (9% of snails examined) and also influenced carrion response.

INTRODUCTION

Parasites in a variety of taxa significantly affect intermediate host behavior in a diversity of habitats (Rothschild, 1962) and these effects have been viewed primarily as parasite adaptations that increase the likelihood of transmission to the next host (*e.g.,* Carney, 1969; Holmes and Bethel, 1972; Moore, 1984). Behavior of animals with respect to nutritional resources in the environment is often studied (*e.g.,* Carr, 1967a, b; Crisp, 1978; McKillup and Butler, 1983) but there has been scant attention to the influence parasites might exert on such behavior, even when parasites are likely to be present [see Etges (1963) and Liebman (1983) for two exceptions]. Likewise, the importance of sex to resource utilization patterns is often ignored.

It is the purpose of this paper to report on a series of experiments carried out to determine the influence of sex and parasitism on carrion response in an important

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Atlantic (and Pacific) coast gastropod. Results indicate that sex and parasitism are indeed important influences on this host's behavior, and that future studies involving this (and probably other) species should take such influences into account. The Cape Henlopen sandflat on the western shore near the mouth of Delaware Bay harbors a large population of the mudsnail, *Ilyanassa obsoleta,* which sustains extensive populations of trematode germinal sacs (Curtis and Hurd, 1983). The attraction of *I. obsoleta* to carrion has been known at least since the work of Dimon (1905). Jenner (1956) surmised that since carrion was relatively scarce it could not be the main source of energy for such an abundant snail. However, it has been shown that carrion is more than an incidental nutritional resource since meat is required for survival, growth, and (at least) female reproduction in this snail (Curtis and Hurd, 1979; Hurd, 1985). Curtis and Hurd (1983) first noted that natural aggregations of *I. obsoleta* on carrion were sometimes predominantly female, and probably not a random draw from the available population. We speculated that differential response of the sexes to resources in the environment could explain some of the extensive spatial heterogeneity we observed in this seemingly homogeneous population. Present results support this by showing that males and females are differentially responsive to carrion. Also, since parasites can profoundly affect intermediate host behavior and there are several reports of the effects of trematodes on *I. obsoleta* ecology, behavior, and physiology (Sindermann, 1960; Vernberg and Vernberg, 1963, 1967, 1968; Stambaugh and McDermott 1969), we suggested that biologists might have to distinguish "apparent" (parasitized) and "functional" (unparasitized) snail individuals in experiments. Results to be presented unequivocally support this suggestion.

Materials and Methods

Experimental sites

Carrion response experiments (CREs) were carried out in Lewes, Delaware, between July 1982 and November 1983. All but one were done on the Cape Henlopen sandflat. The features of this habitat and the *Ilyanassa obsoleta* population there have been described elsewhere (Curtis and Hurd, 1983). To see if carrion response was markedly different in a different habitat, one CRE was done in the nearby Canary Creek Marsh in one of the mosquito ditches where *Ilyanassa obsoleta* was abundant. This site was used in an earlier study (Curtis and Hurd, 1981) and is described there as the "high-marsh-ditch-site."

Experimental design

Approximately two hours (one on either side of low water) were available for each experiment. Before and after that period water tended to be too deep to work effectively. I chose sites on the basis of a steady ebbing current to carry stimulatory substances to the snails, a water depth of 10 cm or less for ease of observation, and an abundance of snails that would neither prove too numerous to dissect nor provide too few snails. Experiments were run throughout the period when snails were active (April–November).

The general plan of each CRE is as follows. Experimental arenas (1 × 5 m) were delimited from the surrounding sandflat with strips of hardware cloth (5 × 5 mm mesh). The hardware cloth fence was buried 3–4 cm in and stood 15 cm above the substratum, and served to exclude all non-arena snails. Arenas were set up with long axis in the direction of the water current. After setting up an arena I collected a control sample to measure frequencies of snails in various conditions of sex and parasitism in the arena population. Five 0.09 m^2 plots were sampled along each longitudinal half of the arena at 1 m intervals for a total of 10 plots. The sampling frame used in this procedure was subdivided with string into 5 × 5 cm squares which I systematically searched by eye and hand for *Ilyanassa obsoleta.* The first 10 snails encountered in each plot were retained separately for later examination. Other snails in a plot were counted but disturbed as little as possible. In cases where at least one but fewer than 10 snails were present in a plot, additional snails adjacent to the sampling frame were collected to make up the sample of 10 for examination. The 100 snails from the 10 plots within each arena served as a control sample for that particular experiment. Mean number of snails per 0.09 m^2 plot was extrapolated to provide an estimate of total snails in the arena. This estimate, minus snails taken as controls, provided an estimate of total snails available to respond.

Carrion used in CREs was either large commercial frozen shrimp or freshly opened hard clams, *Mercenaria mercenaria.* Carrion wet weight was recorded. After collecting the control sample, carrion was placed inside the arena just downstream from the upstream end. Carrion was fixed in place by pinning it to the substratum with a small wooden stake. A responding snail is one that under the experimental conditions moved to the carrion and began to feed. The moment of carrion placement was time zero and snails were collected as they arrived on the carrion for one hour afterward if current direction did not change and if snail activity permitted. Number of snails arriving in each 5-minute interval after time zero was noted. Responding snails were retained for later examination. Air and water temperature were measured. A refractometer was used to measure water salinity to the nearest g/kg.

In the laboratory, snails in responding and control groups were measured for shell height and dissected to determine sex, sexual condition, and presence or absence of parasitism. *Ilyanassa obsoleta* is dioecious and Smith (1980) provides a useful description of its reproductive system. Sexual conditions (following Hurd, 1985) were assigned to females according to presence of an egg capsule in a capsule gland (fec); presence of a capsule gland but absence of an egg capsule (fg); absence of a capsule gland and absence of any indication of a penis behind the right tentacle (f). Females in the fec or fg conditions are usually either reproductive (fec) or potentially so (fg). When out of the reproductive season, and/or when parasitized, females commonly appear in the f condition. Imposex females (Smith, 1980) were noted and recorded. Male sexual conditions were determined by assessing condition of the penis: mp males had a penis with size and shape characteristic of the species, while m males had a reduced penis (usually a bump or scar) and were nonreproductive.

Infection of snails in responding and control groups with trematode germinal sacs was also revealed by dissection. Stunkard (1983) reviewed the trematodes known to infect *Ilyanassa obsoleta* in the Woods Hole, Massachusetts region. His list encompasses (excepting the rare *Pleurogonius malaclemys*) the trematodes of *I. obsoleta* that I have observed in the Lewes, Delaware area; I have followed his nomenclature. As an aid to identification the plates of McDermott (1951) have been used. Species of trematode(s) (if any) infecting each collected snail was (were) recorded.

Analysis of data

Data available from each CRE include: experimental environmental conditions (temperature and salinity); wet weight of carrion used; an estimate of number of snails available to respond; size of each snail; frequencies of snails in various states of sex, sexual condition, and parasitism in a control group; frequencies of the same for snails in a responding group; and number of responders arriving on the carrion in each

5-minute interval of the experimental period. Since an estimate of number of snails available to respond was known, percent snail response could be calculated. Regression analyses were used to reveal significant correlations between percent response and water temperature, salinity, percent controls parasitized, number of snails available to respond, and wet weight of carrion presented.

A goodness-of-fit test was used to test the homogeneity of frequencies of snails in various states of sexuality and parasitism in responding and control groups. Experiments were combined into breeding and nonbreeding sets on the basis of fec presence/absence for this purpose. The test used calculates the statistic U (equivalent to χ^2) which is distributed approximately as Chi-square if expected frequencies are not too small (Powell, 1982). In calculating U ($= \Sigma \frac{(ob - e)^2}{e}$, where ob = observed and e = expected number of snails), I have used actual numbers of snails with various characteristics in the responding group as observed frequencies and used percentages in the control group to derive expected frequencies. If $P < 0.05$, the deviations between observed and expected frequencies were accepted as significant. Lastly, since dispersion of various categories of snails (*e.g.*, females, or snails infected with particular parasite species) could affect experimental results, frequencies of snails with certain characteristics in the 10 control plots were used to calculate within-arena coefficients of dispersion (Sokal and Rohlf, 1981) for a number of snail categories in experiments where none of the control plots was empty of snails (*i.e.*, 11/15 experiments).

RESULTS

Experimental parameters, snail size, and response pattern

Within-arena coefficients of dispersion for certain categories of snails were calculated for experiments where 10 control plots were collected and the category of snail in question was present. In the following list n = the number of experiments from which the mean coefficient of dispersion (CD) for the category of snail was calculated: females (n = 11, CD = 0.78); males (n = 11, CD = 0.7); not infected (n = 11, CD = 1.25); *Himasthla quissetensis*-infected (n = 8, CD = 0.92); *Lepocreadium setiferoides*-infected (n = 11, CD = 0.94); *Zoogonus rubellus*-infected (n = 11, CD = 0.77); *Austrobilharzia variglandis*-infected (n = 9, CD = 0.93). These CDs are all close to one and suggest randomness of distribution within experimental arenas for these major categories of snails. This indicates that composition of responding groups was not systematically affected by nonrandom distribution of snail types relative to carrion position.

Table I presents experimental conditions for each of the 15 CREs carried out in this study. Response was always below, and usually well below, 50%. Percent response is correlated in regression analyses only with water temperature ($r^2 = 0.29$, $P < 0.05$), with colder temperatures associated with reduced percent response.

The majority of snails involved in CREs were 20 mm shell height or more. Mean shell height (all CREs combined) was similar among responders and controls (21.8 mm, S.D. = 2.1 and 22 mm, S.D. = 1.9, respectively). Only 1.8% of all snails examined were 15 mm or less and their proportion was virtually identical among controls (1.7%, n = 1321) and responders (1.9%, n = 790).

Response to carrion was perhaps most frequent early in experiments but occurred more or less steadily throughout. Some CREs were terminated because of flooding currents (8/1/82, 8/7 and 11/5/83) and others (6/14 and 7/30/83) because no more snails were responding. In no CRE were numerous (>5) snails still approaching the carrion at termination, so one hour was sufficient to attract virtually all responsive snails in an experimental arena.

TABLE I

Environmental conditions [air (Ta) and water (Tw) temperatures, salinity (sal)], number of available snails, weight of carrion available, and percent of available snails responding during 15 carrion response experiments with Ilyanassa obsoleta

Date mo/da/yr	Ta °C	Tw °C	SAL g/kg	Carrion* type (g)	Estimated # snails available	Percent snails responding
7/4/82	20	30	22	shr (?)	(?)	(?)
8/1/82	28	27	30	shr (23)	2203	4
8/22/82	19	24	22	shr (15)	400	29
11/13/82	10	12	25	shr (19)	222	1
4/27/83	20	19	17	shr (14)	203	0
6/6/83	22	23	27	clam (24)	306	21
6/14/83	?	22	30	clam (32)	314	2
6/16/83	22	21	25	clam (78)	1311	4
7/20/83	26	35	22	clam (94)	272	30
7/26/83	31	31	28	clam (52)	229	15
7/30/83	22	24	26	clam (45)	140	9
8/7/83	34	33	23	clam (76)	4295	3
8/21/83	27	31	29	clam (51)	194	47
10/22/83	14	16	29	clam (45)	805	8
11/5/83	9	11	23	clam (30)	733	3

* Carrion was either commercial frozen shrimp (shr) or freshly killed *Mercenaria mercenaria* (clam). Wet mass (g) is given.

Sex and parasitism

Overall, 2111 snails were examined. For reference, total numbers in each category of sexual condition (with proportion parasitized) were: fec = 85 (4.7%); fg = 378 (25.4%); f = 724 (91.4%); mp = 295 (41.4%); and m = 630 (85.9%). Proportion of females with imposex features was 2.4%.

Results of individual experiments, broken down by sexual condition and parasitism, are presented in Table II. The marsh CRE (7/26/83) was not obviously unique and has been included with the sandflat CREs. Experiments were grouped for analyses into two categories. Presence of females with egg cases (fec) designates a breeding season CRE. Absence of fecs designates a nonbreeding season CRE. The 4/27/83 CRE was not included in either group for the following reasons. There was no response in this experiment (attempted twice at nearby sites with the same result), and field observations suggest that this had little to do with either sex or parasitism. Water temperature was high enough but salinity was low (Table I) because of recent rains. Snails tend to become inactive at salinities somewhere between 20–15 g/kg (Curtis and Hurd, 1980). Indeed, few if any of the snails in the general area were active at the time so the zero response was attributed to low salinity. In subsequent experiments salinities less than 20 g/kg were avoided.

One purpose of this investigation was to assess the effect of sexual condition on carrion response. As indicated above, some individuals in all sexual condition categories were parasitized. Effect of sexual conditions on carrion response was determined separately for uninfected and infected snails both in and out of breeding season (Table III). (Note: the terms positive, negative, and neutral will be used throughout as indicated below to describe carrion responses.) During the breeding season response of uninfected snails was affected by sexual condition. Females with egg cases responded to carrion more frequently than predicted by controls (positive response) while response fre-

TABLE II

Total numbers and frequencies (%) of Ilyanassa obsoleta *in 5 sexual and 10 parasitism conditions in control (CF = females, CM = males) and responder (RF = females, RM = males) groups from 15 carrion response experiments*

			Percent of total #														
			Sexual conditions					Parasitism									
Date mo/da/yr	Group	Total #	fec	fg	f	mp	m	NI	Hq	Ls	Zr	Av	Ga	HZ	LZ	LG	O
7/4/82	CF	55	7	9	84			20	13	26	20	2		9	7		4
	CM	44				16	84	14	27	23	25			7	5		
	RF	27	22	4	74			33	11	22	17			11	6		
	RM	18				17	83	30	30	19	11			4	7		
8/1/82	CF	60	27	53	20			58	7	2	28	5					
	CM	40				50	50	63	5	8	18	2					4
	RF	50	27	35	38			54	8	11	27						
	RM	35				51	49	40	14	14	26	6					
8/22/82	CF	57		12	88			16	12	26	25	2		12	4		3
	CM	42				29	71	15	33	33	10	2		2		2	3
	RF	61		8	92			8	18	44	11	2		10	7		
	RM	55				11	89		25	42	16		4	7	5		
11/13/82	CF	60		12	88			20	25	20	17	3		12		2	2
	CM	40				50	50	20	28	20	18	5	2	5			2
	RF	2					100		50	50							
	RM	0															
4/27/83	CF	18		17	83			11	22	17	33		11			6	
	CM	22				32	68	18	14	14	32		9	4		4	4
	RF	0															
	RM	0															
6/6/83	CF	33	3	14	84			9	27	24	27			12			
	CM	66				22	77	8	18	17	27	4		11	9		6
	RF	36	3	3	94			3	17	20	33	6		14	6		
	RM	27				19	81	3	14	33	33			11	7		
6/14/83	CF	26	15	27	58			35		35	30						
	CF	14				43	57	36		36	28						

	RF	5	60		40			60		20	20						
	RM	1					100			100							
6/16/83	CF	48	31	48	21			77		2	15	2			2		2
	CM	52				73	27	69		6	17	2	4		2		
	RF	37	48	38	14			76	2	2	16				2		2
	RM	13				62	38	62			31						8
7/20/83	CF	55	5	4	91			11	20	25	36	2		4	2		
	CM	45				4	96	11	9	42	18			13	7		
	RF	46		2	98			15	22	41	13			4	6		
	RM	35				11	89	9	23	29	31			6	3		
7/26/83	CF	41		68	32			78		5	12			2			2
	CM	46				63	37	83		4	13						
	RF	21		52	48			81		5	14						
	RM	14				86	14	71			29						
7/30/83	CF	37		51	49			32		3	27	3	27			3	6
	CM	22				55	45	23			45		18			9	5
	RF	8		50	50			25	37	13	13		13				
	RM	4					100			25	50		25				
8/7/83	CF	50		92	8			74		4	22						
	CM	49				39	61	71	2	6	21						
	RF	91		88	12			77	1	1	21						
	RM	28				46	54	50	7	4	39						
8/21/83	CF	51		12	88			24	14	18	35	8			2		
	CM	49				14	86	16	18	24	34	2			4		2
	RF	47		15	85			36	15	19	17		2	4	2		4
	RM	44				18	82	23	23	18	23		2	5	5	2	
10/22/83	CF	51		8	92			4	12	20	14	8	6	22	2	10	2
	CM	48				6	94	4	23	12	15	4	12	15	6	4	5
	RF	40		10	90			7	25	27	17	3	3	10	3	6	
	RM	24				12	88	8	29	29	13		8	8	4		
11/5/83	CF	58		48	52			15	5	7	53	2	12	2			4
	CM	42				36	64	12	12	10	50	5	2		2		7
	RF	15		80	20			67	7	7	20						
	RM	6				33	67	17	17	17	33	17					

Fec = female with egg case, fg = female with capsule gland, f = female without capsule gland, mp = male with penis, m = male with penis stub, NI = not infected, Hq = *Himasthla quissetensis*, Ls = *Lepocreadium setiferoides*, Zr = *Zoogonus rubellus*, Av = *Austrobilharzia variglandis*, and Ga = *Gynaecotyla adunca*. Frequent double infections (HZ, LZ, LG) are indicated with the generic abbreviations of the species involved. Infrequent single and double infections are grouped under O.

TABLE III

Effect of sexual condition on carrion response of Ilyanassa obsoleta. *Shown are observed (ob) and expected (e) numbers of trematode-uninfected and -infected snails in carrion response experiments compiled into breeding season (A) and nonbreeding season (B) groups on the basis of presence/absence of females with egg cases (fec)*[a]

	Uninfected					Infected[b]			
A	fec	fg	f	mp	m	fg	f	mp	m
ob	42^{+}	23^{0}	8^{0}	26^{0}	7^{0}	10^{0}	118^{+}	13^{0}	84^{0}
e	22.6	29.6	6.4	37.6	9.8	17.1	95.2	13.9	98.8
	U = 22.9, d.f. = 4, $P < 0.001$					U = 10.7, d.f. = 3, $P < 0.025$			
B	fg	f	mp	m		fg	f	mp	m
ob	102^{+}	22^{0}	21^{-}	16^{-}		21	140	23	115
e	72.4	14.7	40.4	33.5		23.9	139.9	34.4	100.8
	U = 34.1, d.f. = 3, $P < 0.001$					U = 6.1, d.f. = 3, $P > 0.05$			

[a] Results of goodness-of-fit tests (U is equivalent to χ^2) are shown which test the null hypotheses that frequencies of snails in five sexual conditions among responders are not different than predicted by frequencies among controls. In significant tests cells with contributions to U of 3.84 or more [significant U ($P < 0.05$) with d.f. = 1] are marked (+) if snails in the category responded significantly more than predicted by controls, (−) if fewer than predicted responded, and (0) if they responded as predicted. See Table II for sexual condition symbols.

[b] Four infected control fecs were merged with the control fgs in the breeding season test.

quencies of other categories were not significantly different from those predicted (neutral response). Response to carrion by uninfected snails after the breeding season was also affected by sexual condition. Whereas females with capsule glands but no egg case (fg) were neutral during the breeding season, they were positive afterward. Uninfected males (mp and m) changed from neutral to negative since they responded significantly less frequently than predicted by controls after the breeding season. Uninfected females with no capsule gland (f) were neutral in both seasons. Among infected snails, sexual condition only affected carrion response during the breeding season, when f females demonstrated a positive response and other categories were neutral. The general result is that depending upon season, sexual condition, and infection presence/absence females tended to respond to carrion in a positive or neutral way while males, depending upon the same factors, tended to be either neutral or negative.

Table IV presents results of goodness-of-fit analyses which tested whether particular parasite species (and absence of infection) affected response of snail hosts to carrion. Three snail groups, consisting of (1) both sexes combined, (2) females only, and (3) males only, were tested for parasite influence both in and out of breeding season. Notably, during the breeding season responses of neither females nor males nor both sexes combined were influenced by parasitism. However, after the breeding season, parasites had a pronounced and unequal effect on carrion response of the two sexes. Uninfected females showed a positive response during this time while uninfected males demonstrated a negative response. Snails infected with *Himasthla quissetensis* were neutral. Both females and males infected with *Lepocreadium setiferoides* showed a positive response. *Zoogonus rubellus* and *Gynaecotyla adunca* infections resulted in a negative response among females but were associated with a neutral response among males. *Austrobilharzia variglandis* infections were inhibitory for both sexes combined and for females.

TABLE IV

Effect of trematode infections on carrion response of Ilyanassa obsoleta *in and out of breeding season*

	Parasitism categories										
	NI	Hq	Ls	Zr	Av	Ga	HZ	LZ	LG	O	U
I											
A	0.31	3.20	0.91	0.36	1.02	n.t.	1.21	0.01	n.t.	6.42	13.44ns
B	2.13^0	2.97^0	12.70^+	6.24	4.84^-	8.28^-	0.39^0	4.90^+	2.62^0	8.45^-	53.52s
II											
A	0.02	1.81	0.64	2.10	n.t.	n.t.	0.00	n.t.	n.t.	0.70	5.27ns
B	15.33^+	1.54^0	4.06^+	9.25^-	5.54^-	8.55^-	2.58^0	n.t.	n.t.	1.50^0	48.35s
III											
A	2.02	1.01	0.79	1.92	n.t.	n.t.	0.13	0.67	n.t.	1.96	8.50ns
B	6.50^-	2.58^0	10.34^+	0.02^0	n.t.	0.03^0	1.38^0	n.t.	n.t.	3.20^0	24.05s

Results for each cell in goodness-of-fit tests and overall U values are shown which test the null hypotheses that frequencies of categories of parasitism among responding snails are not different from frequencies predicted by controls in (A) and out (B) of breeding season. Results from tests on both sexes combined (I), females alone (II), and males alone (III) are shown. See Table III for explanation of +, −, and 0 symbols (n.t. = no test; ns = nonsignificant test, $P > 0.05$; s = significant test, $P < 0.001$) and Table II for parasite symbols.

Multiple infections (frequency among snails examined = 9%) also influenced carrion response, although not many of these categories were individually frequent enough for inclusion in tests (Table IV). *Lepocreadium setiferoides* (positive) and *Zoogonus rubellus* (negative in females) in the same snail produced a positive response (both sexes combined). However, when *Lepocreadium setiferoides* and *Gynaecotyla adunca* (negative in females) occurred together, positive and negative effects apparently cancelled producing a neutral response. The "Other" category includes a variety of infrequent single and multiple infections. Collectively, these produced a negative effect on carrion response (both sexes combined). Included in this category are snails infected with (singles) *Stephanostomum tenue, S. dentatum Diplostomum nassa,* and *Pleurogonius malaclemys;* (doubles) *Himasthla-Austrobilharzia, Himasthla-Gynaecotyla, Lepocreadium-Austrobilharzia, Zoogonus-Austrobilharzia, Zoogonus-Gynaecotyla, Zoogonus-Diplostomum, Austrobilharzia-Gynaecotyla,* and *Gynaecotyla-Diplostomum;* and (triples) *Zoogonus-Austrobilharzia-Diplostomum* and *Lepocreadium-Zoogonus-Austrobilharzia.*

DISCUSSION

Results show that response to carrion by *Ilyanassa obsoleta* is strongly influenced by a number of factors (not necessarily independent) including water temperature (Table I), season, sexual condition, and, not least, infection with trematode germinal sacs (Tables III, IV). Crisp (1978) has shown that recent snail feeding history is also important. Conclusions with respect to effects of sex and sexual condition on carrion response can best be derived from analyses of uninfected snails (Table III) since the confounding effects of parasites are eliminated. Males tend to be neutral during the breeding season and negative afterward. In contrast, females may be neutral (fg and f during, and f after the breeding season), but tend to show positive responses (fec during, and fg after). It is also of interest that sexual condition affected carrion response of infected snails only during the breeding season, when f females showed a positive response (Table III). Why should male and female response to carrion be so demonstrably different? Female reproductive activity (especially production of eggs and egg capsules) is energetically costly (Pechinik, 1979). In another neogastropod group (*Conus* sp.) Perron (1981) found that ova cost 6238 and capsule material 5442 cal/g. Since egg capsules are proteinaceous (Perron, 1981; Sullivan and Maugel, 1984) it follows that need for protein in the diet of females should increase. Hurd (1985) has shown that females require meat for reproductive output. The conclusion seems inescapable that females have an enhanced need for carrion during the breeding season and that this explains the positive response of fec females during that time (fg and f females discussed below).

Influence of trematode parasites is only evident after snail reproductive season and is not the same among males and females. Among females infection with *Himasthla quissetensis* produces a neutral response; infection with *Zoogonus rubellus, Austrobilharzia variglandis,* or *Gynaecotyla adunca* produces a negative response; and being uninfected or infected with *Lepocreadium setiferoides* produces a positive response. Uninfected males, on the other hand, are inhibited rather than stimulated as with uninfected fg females. Males infected with *Z. rubellus* or *G. adunca* are neutral rather than inhibited. Points of male-female agreement are that both infected with *L. setiferoides* demonstrate a positive response and both infected with *H. quissetensis* show a neutral response.

It is apparent that the effects of parasitic infection on this intermediate host are varied, species-specific, and not fully understood. Generally, intermediate host behavior changes of this sort have been interpreted as parasite-induced modifications to increase probability of transmission to the next host (*e.g.,* Holmes and Bethel, 1972; Moore, 1984). In the present case this reasoning seems not to apply because transmission from the snail is by cercariae, and usually to a second intermediate host which may then be ingested by a definitive host (various species of fish or birds in the case of *Ilyanassa obsoleta* trematodes). Other selective forces must be hypothesized to explain observed response modifications. Parasites obtain nutrition from hosts and it is to be expected that host nutritional state will be influenced. Wright (1966) has reviewed pathogenesis of helminths in Mollusca and specific studies of the effects of trematode germinal sacs on marine/estuarine prosobranchs have been made (*e.g.,* Rees, 1934, 1936; James, 1965; Cheng *et al.,* 1983; Sousa, 1983). In *Littorina littorea* germinal sacs block digestive gland tubules and create starvation effects. Even in unblocked regions of the gland glucose, glycogen, glycoproteins, and lipids were all reduced (James, 1965). If a snail is not seriously debilitated by its infection this suggests an explanation for enhanced response to carrion. In this study *Lepocreadium setiferoides*-infected snails, whose protein metabolism is altered compared to uninfected or otherwise infected *I. obsoleta* (Cheng *et al.,* 1983), demonstrated such a response and may serve as an example. The mechanism of such a behavior modification is obscure. The snail may respond as a consequence of simple starvation effects or the parasite may have a nutritional need of its own and secrete an allomone (Holmes and Bethel, 1972) to induce snail behavior. In either case, since parasitized snails are virtually always sterile (Curtis and Hurd, 1983), any adaptive value in the behavior must accrue to the parasite not the snail.

Negative responses on the part of infected snails are most likely explained by debilitating effects of parasites. Cheng and Snyder (1962) reviewed the extensive tissue damage associated with trematode infections in gastropods. Sinderman (1960) proposed that *Austrobilharzia variglandis* infections prevented *Ilyanassa obsoleta* from joining the migration to deeper water (Batcheldor, 1915) at season's end. My observation that snails infected with this species have a negative carrion response (Table IV) supports his contention. Moreover, Stambaugh and McDermott (1969) have shown that parasites (not including *Lepocreadium setiferoides*) make *I. obsoleta* move less often and more slowly than unparasitized controls.

A comparison of Tables III and IV makes interactions between sex and parasitism with regard to carrion response evident. For example, uninfected fg females have a neutral response during but positive response after the breeding season. My tentative explanation for breeding season neutrality is that perhaps the fg females involved had already made their egg cases and had reduced need for carrion (see fec discussion above). However, the postbreeding season positive response probably has more to do with being unparasitized (see NI IIB Table IV) than with being female. The only pronounced postseason fg response occurred in the 11/5/83 CRE (Table II) when water temperature was quite cold (Table I) and it may be presumed that healthy (unparasitized) snails were most able to respond (10/12 responding fgs were unparasitized). One of the most striking results in Table IV is the lack of parasite influence during the breeding season. Infected females in the f condition are an important illustrative point. In season, even though reproductively incompetent, these females nevertheless behaved the same as females in reproductive condition (fec) with respect to carrion (Table III). Out of season, f female carrion response was apparently influenced by other factors (Table III), and Table IV shows that parasites were prominent among these. Effects of parasitism are undoubtedly manifold (Wright, 1971), but whatever their effects it is remarkable that parasitized (sterile) females respond as reproductive individuals at one time of year and as parasitized individuals at another. This suggests some sort of dynamic balance (perhaps chemically mediated) between host and parasite influence on host behavior.

In this study multiple infections occurred in 9% of snails examined and involved a variety of doubles and two triples (Table II and Results). Ewers (1960), Vernberg *et al.* (1969), and Rohde (1981) studied marine/estuarine snails, and Cort *et al.* (1937) and Bourns (1963) studied freshwater snails in this regard. Vernberg *et al.* (1969) studied an *Ilyanassa obsoleta* population in North Carolina and found a multiple infection frequency of 0.02% among 5025 snails. These workers did not examine all snails by dissection and this (and perhaps small and/or large scale spatial heterogeneity as well) probably explains the magnitude of discrepancy between our observed frequencies. Two major points of agreement between this study and that of Vernberg *et al.* (1969) remain, however: *Himasthla quissetensis* and *Lepocreadium setiferoides,* though frequent single infections, never occurred together; and *Zoogonus rubellus* was involved in a large proportion of multiple infections. Dynamics of multiple infections have been considered by several authors (Cort *et al.,* 1937; Ewers, 1960; Bourns, 1963; Lie *et al.,* 1965; Vernberg *et al.,* 1969; Lim and Heyneman, 1972) and it seems certain that these patterns are biologically founded and merit further study.

Where multiple infections are abundant they can affect response to carrion. Indications from this study suggest that contributors to multiple infections can exert contrasting influences. The positive effects of *Lepocreadium setiferoides* apparently override the negative effects of *Zoogonus rubellus* but are neutralized by the negative effects of *Gynaecotyla adunca* (Table IV). This result suggests that outcomes of interactions between parasites (in terms of influencing host responses) in one snail are unique to the species involved, and makes interpretation of host behavior even more complex.

By now effects of parasites on intermediate host individuals or populations have been studied in a number of ecological contexts including terrestrial (*e.g.,* Carney, 1969; Moore, 1984), freshwater (*e.g.,* McClelland and Bourns, 1969; Holmes and Bethel, 1972), and marine/estuarine (*e.g.,* Swennen, 1969; Riel, 1975; Curtis and Hurd, 1983; present study) systems. More than 20 years ago Rothschild (1962) emphasized the impact of parasite-induced modifications of host behavior on almost any study of the host organism. While there is a growing knowledge of parasites and intermediate hosts there is nevertheless a tendency to underestimate the magnitude of influence parasites may exert. Results from the present study on carrion response are complex, and interpretable only because sex and parasitism were measured. A list of studies in which conclusions might have benefitted by incorporating an assessment of parasitism in experimental animals would be very long. Many authors conducting studies involving behavior of *Ilyanassa obsoleta* (or related snails) with respect to carrion and other environmental stimuli have basically ignored the possible influences of sex and parasites on experimental outcomes (*e.g.*, Dimon, 1905; Carr, 1967a, b; Crisp, 1969, 1978; Atema and Burd, 1975; Stenzler and Atema, 1977; McKillup and Butler, 1983). Curtis and Hurd (1983) showed that spatial heterogeneity with respect to sex and parasite frequencies can be very extensive, even in a seemingly homogeneous habitat. The present study shows that effects of sexual conditions and/or parasites on behavior can be substantial. Conclusions from past and future studies should be evaluated in this light.

Our knowledge of marine/estuarine parasites is probably the least among environments (Rohde, 1982). Results presented here and in other studies (cited above) indicate that ecological importance of parasites is to be more than merely suspected, and that biological knowledge will be well served by paying them more attention.

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GASTROPOD TORSION: A TEST OF GARSTANG'S HYPOTHESIS

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ABSTRACT

Torsion occurs in gastropod molluscs as a 180° twisting of the shell and viscera relative to the head and foot of the veliger larva. Garstang (1928, 1929) proposed, and it has since become widely accepted, that torsion functions as a larval defense by allowing veligers to first pull the head into the shell, then sealing the aperture with the foot and operculum.

However, when we offered pre-torsional and torted larvae of the abalone *Haliotis kamtschatkana* Jonas as prey to seven planktonic predator species from four phyla, in only one case was rate of predation reduced on torted larvae. It therefore appears that torsion does not function defensively, indicating that other selective pressures probably maintained this trait in primitive gastropods.

INTRODUCTION

Gastropod torsion is a morphogenic event which occurs during larval life, and results in a 180° rotation of the shell and viscera relative to the head and foot of the veliger. Many authors have speculated about the adaptive value of torsion (Morton, 1958a), but the hypothesis proposed by Garstang (1928, 1929) remains the most widely accepted (Lever, 1979). Because torsion enables a veliger to retract its head and foot into the shell and subsequently seal the aperture with the operculum, Garstang (1928, 1929) suggested that torsion evolved as a larval defense. Garstang (1929) and several others (Yonge, 1947, 1960; Knight and Yochelson, 1958; Morton, 1958a, b; Fretter and Graham, 1962; Morton and Yonge, 1964; Purcheon, 1968; Yonge and Thompson, 1976) have proposed that torsion arose functionally complete through a single mutation. This view remains tenable because no partially torted fossil gastropods have been found (Knight, 1952; Ghiselen, 1966; Lever, 1979), and retains popularity, in part, because of the revival of saltationist evolutionary theory (*cf.* Eldredge and Gould, 1972). The defensive benefits to this torted larva were apparently great enough that it survived to become the progenitor of the Gastropoda. However, Thompson (1967) criticized Garstang's (1929) hypothesis on the grounds that the head and velum are not clearly more vulnerable to attack than the foot, and that opisthobranch veligers do not require torsion to retract completely within their shells (Thompson, 1958, 1967). Jägersten (1972) also suggested that larvae are usually swallowed whole by their predators, and that the ability to retract the head first is probably more important to adults than to larvae.

Because the torted condition persists in most juvenile and adult gastropods, Garstang (1928, 1929) further suggested that torsion in larvae may create maladaptive features for adults. It has been suggested that shell slits, pallial asymmetries and detorsion in opisthobranch gastropods have evolved, at least in part, to correct detrimental features of torsion for benthic existance (Garstang, 1929; Borradaile *et al.*, 1951; Yonge, 1960). Conversely, Garstang's (1929) hypothesis has been modified because it does

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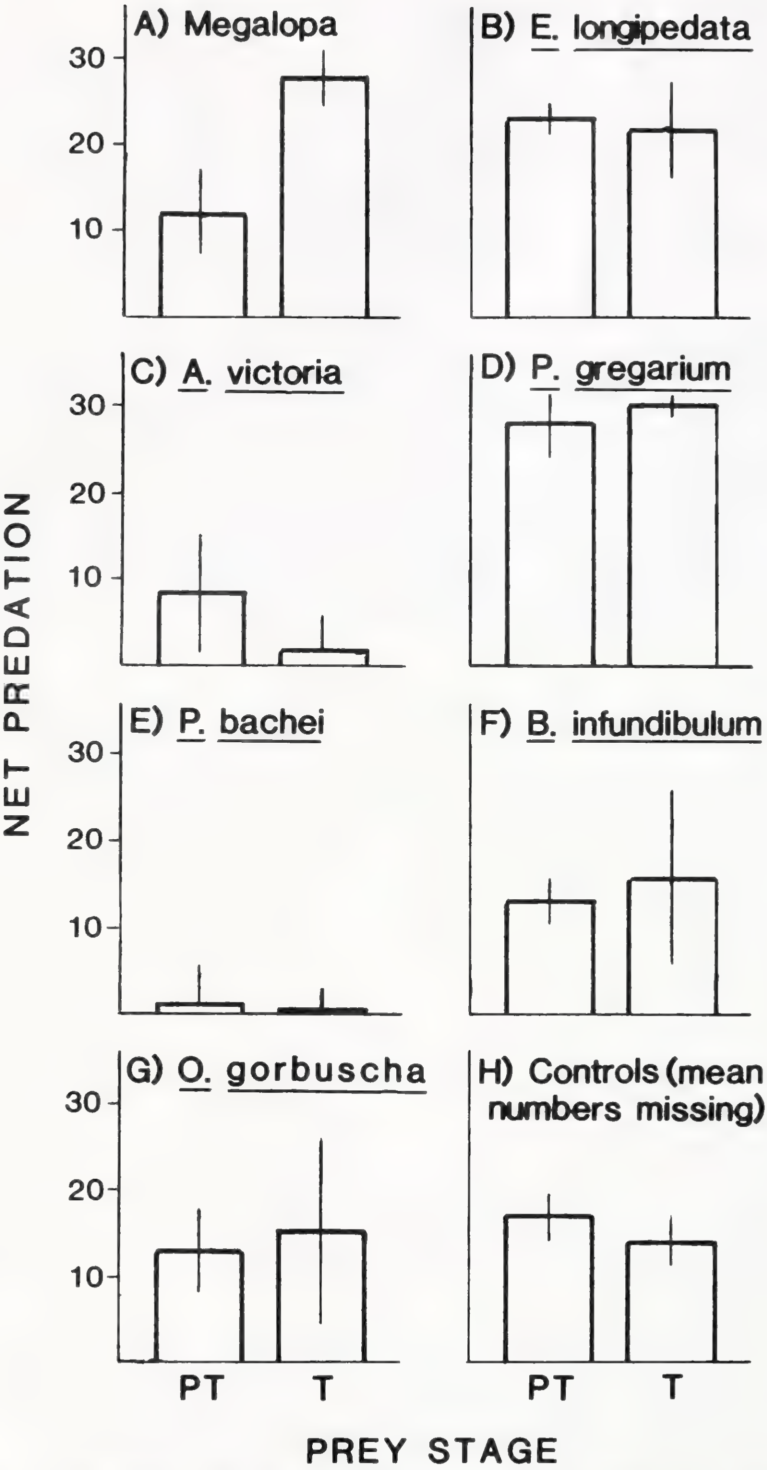

FIGURE 1. "Net predation" upon both pre-torsional (PT) and torted (T) veligers of the archeogastropod *Haliotis kamtschatkana* Jonas by seven species of planktonic predators (A–G; see text for complete descriptions of predators). Bars indicate means ± 1 standard deviation. "Net predation" was calculated by subtracting the mean numbers of veligers missing from control treatments without predators (H) from the mean numbers

not postulate advantages for benthic gastropods. Alterations of mantle position or shell size, weight, coiling, or position have all been suggested to be advantageous consequences of torsion for benthic gastropods (Lang, 1900; Naef, 1911; Yonge, 1947, 1960; Borradaile *et al.*, 1951; Morton, 1958a, b; Allen, 1963; Morton and Yonge, 1964; Ghiselen, 1966; Purcheon, 1968; Jägersten, 1972; Underwood, 1972; Solem, 1974; Lever, 1979; Stanley, 1982). To date, no experimental data have been produced to test Garstang's (1928, 1929) hypothesis, or any other proposed function of gastropod torsion.

This study was designed to test Garstang's (1928, 1929) hypothesis by comparing mortality rates of pre-torsional and torted veligers of the abalone *Haliotis kamtschatkana* Jonas, when offered as prey to an array of seven planktonic predator species from four phyla. The development of *H. kamtschatkana* has been briefly described by Caldwell (1981), and is similar to that reported for other haliotids (Crofts, 1938; Ino, 1953; Carlisle, 1962; Leighton, 1972, 1974). The pre-torsional and torted veligers used in experiments were nearly identical in size and swimming ability, though the torted veligers had undergone only the first 90° of torsion (see Crofts, 1938). At this stage, torted veligers were fully capable of retracting and sealing the shell aperture with the operculum.

MATERIALS AND METHODS

Gametes were obtained according to the methods of Morse *et al.* (1977, 1978), and embryos and larvae were cultured about 48 h (pre-torsional veligers) or 120 h (torted veligers) at 8–10°C in 3 μm filtered seawater plus 50 μg/l each of penicillin G and streptomyosin sulphate. Predators were hand-dipped from surface waters near Friday Harbor Laboratories, Washington, and used in experiments on the day of collection, or fed and maintained a few days in running seawater prior to experiments.

The experiments were similar to those of Pennington and Chia (1984) and Rumrill *et al.* (in press), which documented stage-specific predation upon other larval types. Two experiments were conducted. In the first, pre-torsional veligers were used as prey, and in the second, torted veligers were used. For each experiment 50 veligers were placed into each of 40 one-liter jars containing filtered seawater. Thirty-five of the jars were divided into seven sets of five replicates. Each jar within a set then received a predator species as follows:

(1) five brachyuran megalops larvae (Decapoda);
(2) five *Epilabidocera longipedata* (Copepoda);
(3) one 30 mm diameter *Aequorea victoria* (hydromedusa);
(4) one 12 mm diameter *Phialidium gregarium* (hydromedusa);
(5) one 10 mm diameter *Pleurobrachia bachei* (Ctenophora);
(6) one 20 mm long *Bolinopsis infundibulum* (Ctenophora); or
(7) two 30 mm long *Oncorhynchus gorbuscha* (Pisces).

No predators were added to the five remaining jars which served as controls to measure background prey mortality and handling errors.

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of veligers missing from the treatments with predators. Statistics were calculated with log-transformed raw data; "net predation" was produced for graphic clarity alone. Except for *P. bachei* (E; $P > .05$), all predators ate significant numbers of prey (analysis of variance; $P < .007$ or less). However, only the megalopa (A) and *A. victoria* (C) ate significantly different numbers of either pre-torsional or torted veligers (Student-Newman-Keuls range test; $P < .05$).

During experiments, jars were strapped around the horizontal axis of a grazing wheel (see Landry, 1978; Yen, 1982), which gently stirred the water to keep the prey evenly distributed. Experiments were run for 15 h in a 7:6 h light:dark 9°C coldroom. At the end of experiments, predators were removed and prey were concentrated by siphoning most of the water off through Nitex mesh. Surviving prey were fixed, and counted later. A one-way analysis of variance and a Student-Newman-Keuls range test was calculated with log-transformed data for each predator species. These statistics tested for significant differences between control values and values for treatments with predators, and also for differences in rate of predation upon pre-torsional and torted veligers.

RESULTS

Results of the experiments (Fig. 1) show that with the exception of *Pleurobrachia bachei* ($P > .05$), all predators ate significant numbers of veligers ($P < .007$). *P. bachei* apparently did not consume veligers, though it does eat other planktonic prey in similar experiments (Pennington and Chia, 1984). Rates of predation upon pre-torsional or torted veligers were significantly different only for the megalopa and *Aequorea victoria* ($P < .05$). The megalopa consumed more torted veligers while *A. victoria* ate more pre-torsional veligers.

DISCUSSION

Except for *Aequorea victoria,* no predator species ate significantly fewer torted than pre-torsional veligers. Moreover, any advantage torsion confers to veligers against predators such as *A. victoria* is apparently offset by increased vulnerability to predators such as megalopa, and the apparent effectiveness of torsion against *A. victoria* is not general for hydromedusae, because *Phialidium gregarium* ate nearly equal numbers of both stages of prey. Our results thus do not support Garstang's (1928, 1929) hypothesis concerning the defensive value of torsion in veligers.

We have not determined why torted veligers, which have developed an operculum and can seal themselves within their shells, were as vulnerable to predation as pre-torsional veligers. Shells of larval molluscs have often been suggested to serve defensively, and several authors have even suggested that veligers can pass unharmed through the guts of their predators (Morton and Yonge, 1964; Yonge and Thompson, 1976; others reviewed by Mileikovsky, 1974). Torted veligers placed on a glass slide usually withdrew upon being disturbed for only a few seconds; perhaps this intermittent retraction was insufficient to deter predators that swallow prey whole. Empty veliger shells were often found within the manubria of *P. gregarium* at the end of experiments. Shell fragments were also commonly found within jars that had contained crustacean predators. If the crustacean predators broke shells prior to ingestion, retraction probably did not protect the larva within. Mileikovsky (1974) suggested that if veligers do pass intact through the guts of their predators, they are usually so entangled in mucous and feces that they rarely survive in any case.

The present study provides the first data to test any hypothesis concerning the adaptive value of gastropod torsion. It might be argued that over evolutionary time, small (and therefore undetected by us) decreases in rate of predation due to torsion would provide sufficient selective pressure to maintain the trait. While this argument is valid, Garstang (1928, 1929) and several recent authors (Yonge, 1947; Knight, 1952; Knight and Yochelson, 1958; Morton, 1958a, b; Morton and Yonge, 1964; Purcheon, 1968; Yonge and Thompson, 1976; Stanley, 1982) have proposed that a single mutation caused torsion in a larval pre-gastropod whose fitness became so enhanced that it became the progenitor of the Gastropoda. Our results indicate that the defensive benefits of torsion for veligers, if any, are probably insufficient to foster the foundation of the Gastropoda from a mutation in a single veliger. Our results do support the suggestions of Thompson (1967) and Jägersten (1972), both of whom thought that torsion does not function effectively as a larval defense. It nevertheless remains conceivable that torsion confers other benefits to veligers; for example, torsion might aid in swimming (Underwood, 1972), and it is also possible that torsion protects veligers from adverse physical conditions. Pre-torsional veligers do retract within their shells, though they lack opercula.

It is also possible that untested or extinct predators are deterred by torsion while the predators we used are not. Although our selection of predator species was necessarily limited, we attempted to choose a diverse array of predators that are both common in neritic plankton and which feed by different mechanisms (see Pennington and Chia, 1984). Ctenophores, medusae, crustaceans, and planktivorous fish meet these criteria, though it remains problematic whether a predator such as *O. gorbuscha* had analogues in Cambrian seas. Though we cannot eliminate the possibility that extinct predators provided the selective pressure for torsion in veligers, there is little factual basis to support such an argument.

In contrast to Garstang's (1928, 1929) hypothesis, most other hypotheses regarding torsion postulate advantages during metamorphosis or for juveniles and adults (Lang, 1900; Naef, 1911; Yonge, 1947, 1960; Borradaile *et al.*, 1951; Morton, 1958, 1979; Allen, 1963; Morton and Yonge, 1964; Ghiselen, 1966; Purcheon, 1968; Jägersten, 1972; Underwood, 1972; Solen, 1974; Lever, 1979). If torsion is not a larval adaptation, one or a combination of these hypotheses probably explains its evolution within the Gastropoda. However, the selective pressures that have been suggested to favor torsion will remain speculative until further experimental work is conducted to examine the functional implications of torsion for gastropods.

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INTERACTIONS AMONG TEMPORARY AND PERMANENT MEIOFAUNA: OBSERVATIONS ON THE FEEDING AND BEHAVIOR OF SELECTED TAXA

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ABSTRACT

Meiofauna diets and behavioral patterns are relatively unknown despite the fact that in any system, predatory relationships and behavioral responses may play an important role in determining community structure. Therefore, observations on food preferences, feeding behavior, and encounter interactions of members of a meiofauna assemblage were made in the laboratory in dishes of natural sediment. The diets and behavior of two turbellarian species, *Neochildia fusca* and *Archiloa wilsoni* were examined in detail. Both are predators and both feed on a variety of other taxa, including the temporary meiofauna. Based upon the results of these experiments, a potential food web was constructed among the temporary and permanent meiofauna. The behavioral responses of these turbellarians and other members of the meiofaunal assemblage at times of encounter were also observed, categorized, and quantified. Implications of these behaviors are discussed.

INTRODUCTION

The meiofauna represent a very abundant and potentially important component of marine soft-sediment communities and yet knowledge about the feeding habits of and behavioral interactions among the meiofauna are scarce. The food preferences of some microbial-feeding harpacticoid copepods (Lee *et al.,* 1977; Rieper, 1978, 1982; Vanden Berghe and Bergmans, 1981) and nematodes (Tietjen and Lee, 1977) are partially known from experiments using radiolabeling techniques. Information about turbellarian diets arises mostly from remains visible in the guts of occasional animals (Straarup, 1970, and references therein), although a few largely anecdotal observations have been reported (Meixner, 1938; Pawlak, 1969; Straarup, 1970). For many turbellarian species, gut contents are unidentifiable, or items in the gut (such as diatom frustules) may have a secondary origin (*i.e.,* may have been present in the gut of the prey species consumed). Systematic observations of the feeding behavior of and the various predatory and aggressive behavioral interactions among the meiofauna are almost completely lacking.

In any system of interacting species, species recognition and differential behavioral responses at times of encounter should play a role in establishing and maintaining patterns of co-existence. Therefore, the study of behavioral interactions and natural history of the fauna is extremely important, especially since interpretations of manip-

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ulative field experiments often rest heavily on assumptions about the natural history and behavior of the manipulated species (Dayton, 1979; Peterson, 1979). If the manipulated species is known to be a predator on another species, then a reduction in the abundance of that prey species seems easily interpretable. However, behavioral avoidance of the manipulated species by the prey species could equally well explain lower densities of this prey species in experimental plots. Without some independent observation on mechanism, it is impossible to tell if the negative result of a manipulation is due to predation, behavioral avoidance, competition, or physical disturbance.

Therefore, observations on the food preferences, feeding behavior, and encounter interactions of members of a meiofauna assemblage were undertaken. Because the meiofauna are both small and live in the sediments, field observations are difficult. Thus laboratory experiments were designed to get initial information on some common meiofaunal taxa. Since so little is known about the diets of turbellarians, and yet turbellarian predators could be feeding on both permanent and temporary meiofauna (*i.e.*, the juvenile macrofauna, as defined by McIntyre, 1969), major emphasis was placed on determining the diets and behavioral patterns of two turbellarian species common in muddy-sand habitats, an acoel, *Neochildia fusca* (Bush, 1975), and a proseriate, *Archiloa wilsoni* (Stirewalt *et al.*, 1940). The behavioral responses of other assemblage members to encounter with these two turbellarian species were also observed.

MATERIALS AND METHODS

Sediment was collected from several shallow-water muddy-sand sites in Bogue and Back Sounds, North Carolina, but principally from Tar Landing Bay, a shallow-water embayment on the southeast end of Bogue Sound (34° 42′ N, 76° 42′ W). The surface 2 to 3 cm of sediment was collected and transported back to the laboratory. Sediment was then spooned into flasks and mixed with a $MgCl_2$ solution isotonic to seawater (30 ppt), shaken, and allowed to sit for 15 minutes. Then the flasks were shaken again, and the supernatant decanted through a 63 μm sieve. The material collected on the sieve was rinsed gently with seawater, and the sieve placed in a Petri dish with a small amount of seawater. After at least 1 hour, sieves and Petri dishes were examined under a dissecting microscope and experimental animals were removed by pipette.

Feeding experiments

Experimental animals were placed into culture dishes (3 cm diameter) which contained approximately 5 ml of 63 μm filtered natural seawater and approximately 1 ml of sediment in the center of the dish. Seawater in each dish was changed daily by pipette (under the microscope). Combinations of individual meiofauna and their suspected prey were established. Each combination was checked twice daily under the dissecting microscope to determine if the prey had been eaten. Animals usually stayed in the center of the dish, and could be easily located by spreading the sediment grains apart with a dissecting needle. As soon as the prey was eaten, or if, after three successive days, no predation had occurred, the trial was terminated.

Combinations of *Neochildia fusca* and *Archiloa wilsoni* with their suspected prey were emphasized. Unless otherwise stated, only robust, active individuals were used in the experiments. Some specific trials were made with wounded animals (either the body wall was pricked with the dissecting needle, or the shell was broken), to see if there was a greater response to prey in this condition.

Interaction observations

Interactions between meiofauna (both permanent and temporary, *i.e.*, the juvenile macrofauna) were observed under two sets of conditions.

Under the dissecting microscope (observations from above). Specific pairs of meiofauna were placed in culture dishes, set up in the same way as in the feeding experiments, or whole assemblages were placed in Petri dishes with a layer of sediment several sand grains thick. The majority of the observations were made on animals in the Petri dish situation. Fields of view in the center of the Petri dish were haphazardly located until an individual was observed. This individual was then observed until it disappeared under the sediment surface again or 30 min had passed. Then a new field of view and a new individual were located to prevent making too many observations on the same individual. Usually, this was not a problem, as animals disappeared from view long before the half hour was over. After several such observations, major encounter types and responses were put into categories and the frequency of each response to a particular type of encounter was recorded. Truly unique behaviors also were recorded separately.

Under the inverted microscope (observations from below). Again, whole assemblages were placed in Petri dishes with enough sediment to cover the dish completely with a layer of sediment several sand grains thick. Since most meiofauna are positively geotactic (Boaden, 1977) and burrow down into the sediments, this was an attempt to observe the animals in as nearly a natural condition as possible. A red filter was placed over the light source to minimize light interference with behavior. Observations were made in the same manner as under the dissecting microscope.

More than 500 hours were spent observing these animals, about 400 from above, and 100 from below. A total of more than 1600 interactions were witnessed. Observations from above and below were combined in all the following tables.

Observations on undisturbed sediment cores

Square cores (10 cm by 10 cm) were taken with a coring box which fit into a freezer box with the same dimensions. Cores were covered, transported back to the laboratory and placed in aquaria with running seawater, uncovered, and left for approximately 12 hours. Then an individual core was placed into a smaller aquarium where the surface of the core was covered by approximately 3 cm of seawater and over which a dissecting microscope was mounted on a swing arm. Again fields of view were located haphazardly, and no individual was watched for more than one half hour. Observations were made on the animals visible or appearing at the sediment surface. All observations were timed and all animal movements were recorded.

RESULTS

Feeding experiments

Both *Neochildia* and *Archiloa* did eat a wide variety of the prey species offered, including both temporary and permanent meiofauna (Table I). But both also could be very selective in some instances, for example neither would eat the maldanid *Axiothella mucosa,* but both would eat *Clymenella torquata. Archiloa* fed much more readily on nematodes and copepods than *Neochildia* did. Neither ate bivalves, and *Neochildia* would not eat juvenile amphipods. The percentage eaten in Table I includes both animals completely consumed and those where one third or more of the body was missing. These animals usually died within 24 hours. Table II summarizes these incidences of partial predation. *Archiloa* was much more prone to eat only part of its

TABLE I

The results of the feeding experiments with Neochildia fusca *and* Archiloa wilsoni

	Neochildia fusca		*Archiloa wilsoni*	
Prey species	n	% eaten	n	% eaten
Temporary Meiofauna				
Spionidae				
Streblospio benedicti				
7–12 setigers	5	100	5	80
13–18 setigers	11	73	12	58
>18 setigers	6	33	6	33
Polydora sp	2	50	3	0
Prionospio sp	3	100	3	67
Capitellidae				
Mediomastus sp	3	100	3	100
Heteromastus filiformis	4	100	3	67
Capitella sp	3	67	3	67
Cirratulidae				
<10 setigers	5	60	5	40
>10 setigers	2	50	5	40
Orbiniidae	5	80	3	33
Maldanidae				
Clymenella torquata	4	50	3	33
Axiothella mucosa	3	0	3	0
Terebellidae	4	25	4	25
Nereidae				
<7 setigers	4	50	3	0
8–12 setigers	4	0	6	50
>12 setigers	3	0	3	0
Nephtyidae	3	33	2	0
Hesionidae	3	0	3	0
Syllidae				
<8 setigers	3	33	3	0
>8 setigers	3	0	3	0
Arabellidae				
Drilonereis magna	not tested		2	0
Oligochaeta				
<1 mm	6	67	7	86
> 1 mm	4	0	4	50
Bivalvia	4	0	6	0
Amphipoda	3	0	4	50
Permanent Meiofauna				
Nematoda	6	0	9	55
Copepoda				
Enhydrosoma sp	4	0	9	67
Others	8	25	6	60
Turbellaria				
Neochildia fusca	6	0	9	0
Archiola wilsoni	9	89	6	0
Convoluta sp	7	57	7	71
Macrostomum beaufortensis	2	50	3	0
Species A	3	67	3	33
Species B (acoel)	3	33	3	67
Ostracoda	2	0	3	0
Gastrotricha				
Turbanella	not tested		2	0

n = the number of trials with that potential prey taxon.
% eaten = the percentage of trials in which *Neochildia* or *Archiloa* consumed the prey.

TABLE II

Incidences of partial predation by Neochildia fusca *and* Archiloa wilsoni

Neochildia fusca	Partial predation	Total no. eaten	*Archiloa wilsoni*	Partial predation	Total no. eaten
Oligochaeta >1 mm	1	1	*Streblospio benedicti* >18 setigers	1	2
Terebellidae	1	1	Oligochaeta >1 mm	1	6
Nephtyidae	1	1	Capitellidae *Heteromastus filiformis*	1	2
Orbiniidae	1	4	Cirratulidae >10 setigers	1	2
			Nereidae 8–12 setigers	3	4
			Nematoda	2	5

The partial predation column represents the number of times where less than the whole animal was consumed. The total no. eaten column represents the total number of individuals eaten in the feeding experiments.

prey than was *Neochildia*. Also *Archiloa* ate almost every wounded animal which was offered to it (Table III), whereas *Neochildia* did not eat a single wounded animal.

Behavioral observations

Neochildia fusca. Neochildia fusca is a relatively large (0.1–3 mm in length) elongated-oval shaped turbellarian with a ventral mouth in approximately the center of the body. *Neochildia,* like all acoels, does not have a true gut, but rather a central digestive parenchyma. *Neochildia* moves rapidly and easily through the sediment. It employs several different strategies in feeding. The first is a "sit and wait" strategy. The posterior portion of the body is anchored down in the sediment and the anterior one half to two thirds of the body is arched up above the sediment with the sides of the body folded slightly underneath and inward. Bush (1975) also observed this behavior and as she notes, it makes the animal an excellent trap for passing prey. An animal moving under the raised end of the turbellarian is quickly pounced upon. The cupped portion of *Neochildia*'s body serves to hold the prey from escaping as it is quickly pushed and sucked up into the mouth.

TABLE III

Incidences of predation on wounded animals

Prey species	*Neochildia fusca*	*Archiloa wilsoni*
Streblospio benedicti 13–18 setigers	0	3
Terebellidae	0	3
Copepoda	0	4
Bivalvia (broken shell)	0	2
Oligochaeta	0	3

Streblospio, terebellids, copepods, and oligochaetes were pricked with a dissecting needle. The bivalve shells were broken with forceps. Four replicates of each taxa were offered to each of the two turbellarians.

TABLE IV

Response of Neochildia fusca *to encounter with a variety of other taxa*

Other taxa	Encounter type	Response of *Neochildia fusca*: Violent recoil	Stop, back away	Move over	Attack
Streblospio benedicti, sm.	Head on	0	4	9	2
	Side on	0	2	10	7
Streblospio benedicti, med.	Head on	0	7	6	3
	Side on	0	1	9	7
Streblospio benedicti, lg.	Head on	1	6	4	1
	Side on	0	3	8	5
Polydora spp.	Head on	0	7	3	0
	Side on	0	4	7	4
unmetamorphosed spionid larvae		2	5	0	0
Capitellidae	Head on	1	4	3	1
	Side on	0	2	6	3
Cirratulidae	Head on	2	4	1	0
	Side on	0	6	5	3
Orbiniidae	Head on	1	5	2	0
	Side on	0	4	7	2
Clymenella torquata	Head on	0	3	3	0
	Side on	0	2	5	1
Axiothella mucosa	Head on	0	4	2	0
	Side on	0	5	7	0
Terebellidae	Head on	4	3	1	0
	Side on	0	4	3	1
Nereidae, sm.	Head on	0	1	7	0
	Side on	0	1	8	1
Nereidae, med.	Head on	2	6	7	0
	Side on	0	8	9	0
Syllidae, sm.	Head on	0	2	5	0
	Side on	0	1	7	1
Syllidae, med.	Head on	1	6	2	0
	Side on	0	5	6	0
Glycera sp.	Head on	1	5	0	0
	Side on	0	4	2	0
Oligochaeta	Head on	0	4	8	1
	Side on	0	3	10	5
Bivalvia		0	3	5	2
Nematoda	Head on	0	3	1	0
	Side on	0	4	8	0
Copepoda	Head on	0	6	3	0
	Side on	0	4	7	1

TABLE IV (*Continued*)

Other taxa	Encounter type	Response of *Neochildia fusca*			
		Violent recoil	Stop, back away	Move over	Attack
Neochildia fusca	Head on	14	0	0	0
	Side on	12	3	0	0
Archiloa wilsoni	Head on	0	3	5	6
	Side on	0	1	8	12
Convoluta sp.	Head on	0	3	5	0
	Side on	0	3	6	2
Ostracoda		0	2	5	0

See text for more complete descriptions of encounter type and response categories. Sm., med., lg. refer to the sizes listed in Table I.

Other strategies are more active. *Neochildia* often swims along the surface of the sediment, again with the anterior end of the body slightly raised and cupped inward. It also moves through the sediment in a flattened position. When a prey is encountered on its side or from the rear, *Neochildia* very rapidly moves over it or pins it to a sand grain, again cupping its body around the prey to prevent escape. Events of partial predation usually occurred with larger, vermiform prey. The victim's body was broken off when there appeared to be no more room in *Neochildia*'s digestive parenchyma. However *Neochildia*'s central parenchyma appeared to be very extensible, and *Neochildia* ate many animals as big or slightly bigger than itself.

Encounter types were put into two categories, head on, and side on. Head on is defined as the meeting of the animal being observed (in this case *Neochildia*) and the other animal on the anterior most one fourth of the body (for turbellarians and nematodes) or the part of the body defined as the head (for polychaetes, oligochaetes, and copepods). Side on is defined as the meeting of the head of the animal being observed with the lower three fourths of the body of the animal it encounters.

Responses of the animal under observation were put into four categories; (1) violent recoil, (2) stop and back away, (3) move over or under, and (4) attack. For *Neochildia,* these categories are further defined as follows: (1) violent recoil—as soon as body contact is made between the two animals, *Neochildia* immediately and violently draws back by very abruptly contracting the muscles in the anterior end of the body. This is immediately followed by a change in direction and rapid movement away from the animal it just encountered. (2) Stop and back away—when body contact is made, *Neochildia* quickly stops forward motion, then moves backwards and away from the animal it encountered. When forward motion ceases, the body does not contract as it does in category one. (3) Move over or under—*Neochildia* simply changes the plane of motion very slightly and moves over or under the animal it encountered. (4) Attack—*Neochildia* may back off for a fraction of a second, but then rapidly moves over the animal it encountered, cups its body around it, and then consumes, or tries to consume all or part of it.

Table IV summarizes *Neochildia*'s responses to both types of encounter with a wide variety of other taxa. The violent recoil reaction never occurred when *Neochildia* encountered the other individual on the side except in the case of *Neochildia-Neochildia* interactions. This strong intraspecific avoidance reaction was more pronounced than *Neochildia*'s reaction to any other species. With all taxa, *Neochildia* was much more likely to attack and eat an individual if it was encountered from the side rather than head on.

The two attacks on bivalves were both unsuccessful. In both cases, *Neochildia* encountered the foot of the bivalve and then cupped its body around the clam. However, the bivalve quickly withdrew its foot and closed its shell before *Neochildia* could ingest any clam body parts, and *Neochildia* did not try to engulf the whole animal, shell and all. Once the bivalve was closed, *Neochildia* let go of it and moved on.

Archiloa wilsoni. Archiloa is slightly smaller than *Neochildia* (0.1–2 mm length) and thinner with a pointed tail. The mouth, with a long, cylindrical plicate pharynx, is located about two thirds of the way down the body, opening on the ventral side. It also moves readily and rapidly through the sediment. The feeding behavior of *Archiloa* was observed much less often than that of *Neochildia* even though more individuals of this species were observed. It rarely ate the first day after extraction. In the feeding experiments, its prey, with few exceptions, were only found missing on the second or third day. Initially some animals were observed until the sixth day. However if *Archiloa* had not eaten by day 3, it never ate. By day 4, it appeared emaciated and behaved abnormally.

The feeding behavior of *Archiloa* was similar with all types of prey. After encountering a potential prey, *Archiloa* would swim along side of it, quickly evert its pharynx, thrust it into the victim, and suck the animal, or only the internal organs and fluids, into its own digestive cavity. Copepods were attacked on the ventral side, between the legs. Usually the carapace was not ingested. Several times with larger polychaetes, only a portion of the body was consumed before the animal broke away or was released by *Archiloa.* This must have been the case with the victims of partial predation in Table II. Once with a larger spionid and twice with larger oligochaetes, *Archiloa* would feed for a minute or two, then begin to move through the sediment with the victim still only partly ingested, but still tightly held by the pharynx. With one oligochaete, a few minutes later *Archiloa* stopped and ingested a little more of its victim, then started moving again. Eventually, in all three cases, the sand grains pulled what remained of the prey away from the pharynx, and *Archiloa* kept going without it.

Archiloa readily preyed on wounded animals (Table III). This was first observed with a copepod, and subsequently with another copepod and an oligochaete. In all three of these cases, within 5 minutes, *Archiloa* swam over to the injured individual, moved along side of it, then quickly attacked and ate it.

Encounter types and categories of responses are defined for *Archiloa* as they were for *Neochildia,* except for attack. In an attack, *Archiloa* swims along side the encountered animal, and rapidly everts the pharynx trying to thrust it into its victim to consume all or part of it.

Table V summarizes *Archiloa*'s responses to encounter with other taxa. *Archiloa* showed the violent recoil reaction to a much wider array of taxa than did *Neochildia.* Again, however, this response was much more common in head on encounters than in encounters to the side. The response of *Archiloa* to intraspecific encounters most frequently yielded the violent recoil reaction, but not as overwhelmingly as *Neochildia*'s reaction to intraspecific encounter. In all encounters of *Archiloa* with *Neochildia,* the response was the violent recoil. Several times after an encounter of this type, *Neochildia* quickly turned, chased, and attacked *Archiloa.* Three of *Archiloa*'s attacks on *Streblospio* (2 medium, 1 large) were not completed. The pharynx was everted and touched *Streblospio*'s body, but there appeared to be no suction and the animal was quickly released.

TABLE V

Responses of Archiloa wilsoni *to encounter with a variety of other taxa*

Other taxa	Encounter type	Response of *Archiloa wilsoni*			
		Violent recoil	Stop, back away	Move over	Attack
Streblospio benedicti, sm.	Head on	0	12	17	2
	Side on	0	10	14	7
Streblospio benedicti, med.	Head on	2	17	13	1
	Side on	0	12	11	6
Streblospio benedicti, lg.	Head on	3	9	8	0
	Side on	0	6	5	4
Polydora spp.	Head on	0	3	5	0
	Side on	0	4	6	2
Unmetamorphosed spionid larvae		4	8	0	0
Capitellidae	Head on	3	5	4	1
	Side on	0	3	5	4
Cirratulidae	Head on	5	6	0	0
	Side on	0	3	5	2
Orbiniidae	Head on	3	6	1	0
	Side on	1	2	4	2
Clymenella torquata	Head on	2	3	1	0
	Side on	1	4	1	1
Axiothella mucosa	Head on	3	4	2	0
	Side on	1	3	4	0
Terebellidae	Head on	4	4	1	0
	Side on	2	5	4	1
Nereidae, sm.	Head on	1	2	4	0
	Side on	0	2	5	0
Nereidae, med.	Head on	3	4	3	0
	Side on	0	2	4	1
Syllidae, med.	Head on	1	4	1	0
	Side on	0	3	4	0
Glycera sp.	Head on	1	5	0	0
	Side on	0	3	2	0
Oligochaeta	Head on	0	7	2	0
	Side on	0	1	4	5
Bivalvia		0	1	8	0
Nematoda	Head on	0	2	1	0
	Side on	0	4	6	1
Copepoda	Head on	0	6	1	0
	Side on	0	8	3	2
Neochilda fusca	Head on	18	0	0	0
	Side on	20	0	0	0
Archiloa wilsoni	Head on	7	6	0	0
	Side on	8	3	0	0
Convoluta sp.	Head on	0	2	5	0
	Side on	0	1	6	2
Ostracoda		0	3	7	0

See text for more complete descriptions of encounter type and response categories. Sm, med., lg. refer to the sizes listed in Table I.

Enhydrosoma sp. *Enhydrosoma* is frequently the most abundant harpacticoid copepod in the Tar Landing Bay assemblage. It is a small (200–500 μm) vermiform copepod which moves easily and quickly between and around sand grains. It is assumed to be a deposit feeder. Some observations were made (Table VI). Encounter types are as described for *Neochildia.* Response categories differ as follows. (1) Violent recall—no perceptable stopping is observed. *Enhydrosoma* nearly instantaneously changes direction 180° and rapidly swims away from the encountered individual. (2) Stop and move away—All forward motion ceases. Then *Enhydrosoma* changes direction and swims away. The primary difference between this response and the first response is in the presence of the stop and the speed with which the whole reaction takes place. (3) Move over or under—as with *Neochildia,* the plane of movement is slightly altered, and *Enhydrosoma* simply swims over, under, or around the encountered animal. (4) Circle—the response category is unique to *Enhydrosoma.* It simply moves off the animal, then swims around it in a circle several times, appearing occasionally to touch it with the legs or antennae.

The circle response was exhibited particularly to *Streblospio* and bivalves (Table VI). In the case of *Streblospio,* the response seemed to be elicited by encounter with the partial tube of *Streblospio* as much as it was by an encounter with the animal itself. The violent recoil response was only exhibited in six cases, all of these head on

TABLE VI

Responses of Enhydrosoma *sp. to encounter with a variety of other taxa*

		Response of *Enhydrosoma* sp.			
Other taxa	Encounter type	Violent recoil	Stop, move away	Move over/under	Circle
Streblospio benedicti	Head on	2	1	1	0
	Side on	0	2	5	4
Capitellidae	Head on	0	2	3	1
	Side on	0	0	4	3
Axiothella mucosa	Head	0	3	0	0
	Side on	0	1	1	2
Oligochaeta	Head on	2	1	1	0
	Side on	0	3	0	0
Bivalvia		0	0	1	4
Nematoda	Head on	0	3	0	0
	Side on	0	1	9	0
Copepoda, other	Head on	0	4	3	0
	Side on	0	3	4	0
Small Turbellaria	Head on	0	2	5	0
	Side on	0	1	6	0
Neochildia fusca	Head on	1	4	2	0
	Side on	0	4	4	0
Archiloa wilsoni	Head on	1	3	1	0
	Side on	0	3	3	0

See text for more complete descriptions of encounter type and response categories.

encounters. While major attention was focused on *Enhydrosoma* in these observations, some note was taken of what the other animal did after *Enhydrosoma* encountered it. Bivalves quickly closed their shells. Particularly when the circle response was initiated, *Streblospio,* capitellids, and *Axiothella* withdrew or tried to move away from *Enhydrosoma.* Nematodes always responded by moving away.

Nematodes. Identification of nematodes was not undertaken. Therefore it is not known how many species are included in this group. The only criterion used in selecting individuals to observe was reasonably large size. These results are only intended to indicate the general breadth of nematode behavior. Encounter types and response categories are as described for *Enhydrosoma,* except that the direction change in violent recoil is rarely as great as 180°.

Nematodes move through the sediments by both pushing on sand grains and moving sand grains from their directional paths (Pitcher, 1975). When there is very little sediment, nematodes flex their muscles, but do not move in any direction, and simply whiplash back and forth. This behavior was observed frequently in culture dishes, and did sometimes result in damage to individuals of other species (*i.e.,* tentacle loss in spionids). However when sufficient sediment is present, muscle contraction results only in the nematode moving quickly through the sediment. Therefore only reactions seen while following nematodes in sufficient sediment in Petri dishes are reported here.

Table VII summarizes the responses of nematodes to other taxa. Like the two turbellarians and *Enhydrosoma,* the violent recoil response most often followed head on encounters. Unlike the two turbellarians, the nematode-nematode encounter did not yield the violent recoil reaction. In most cases the two nematodes simply moved past each other, or stopped, changed direction, and moved away from each other. In general, the other taxa responded to nematode encounter by movement away.

Nematodes are known to excrete mucus (Riemann and Schrage, 1978). Some secrete copious amounts which very visibly binds the sediment and detritus together in long strings. This mucus apparently remains sticky for quite awhile after it has been secreted, and I have often observed that other meiofauna become tangled in this mucus. In four cases, these tangled individuals were encountered by one of the two turbellarians predators, and became easy prey, as they could not swim away.

Temporary meiofauna. Reactions of temporary meiofauna to encounters with *Neochildia* and *Archiloa* also were observed (Table VIII). Because many of these taxa are not mobile, following an individual and recording its responses to encounter with other individuals was not always feasible. Therefore animals were first observed and put into mobility categories. Sedentary species never moved more than half a body length. The two maldanid species (*Clymenella torquata, Axiothella mucosa*) would construct partial tubes around themselves and usually only moved within the confines of these tubes. The occasionally mobile taxa usually stayed in one place, but would occasionally move away a short distance, and then remain stationary again. The spionids (*Streblospio benedicti* and *Polydora* sp.) also constructed tubes around themselves and would move their anterior ends in and out of the tube, but only occasionally would leave their tubes and move to a new area. This was usually in response to some outside disturbance. Sedentary and occasionally mobile taxa were observed in place. Since they rarely ran into other individuals, their responses to other animals running into their head region were also recorded. Mobile taxa moved actively through the sediments and were followed through the sediments in the same manner as before.

Responses of these temporary meiofauna to *Neochildia fusca* and *Archiloa wilsoni* again were put into categories. The categories are defined as follows: (1) withdraw—the animal rapidly draws back into its tube in the case of the tube dwellers, or simply

TABLE VII

Responses of nematodes to encounter with a variety of other taxa

		Response of Nematoda		
Other taxa	Encounter type	Violent recoil	Stop, back away	Move over
Streblospio benedicti	Head on	2	3	0
	Side on	2	5	3
Capitellidae	Head on	2	3	0
	Side on	0	3	5
Terebellidae	Head on	3	3	0
	Side on	0	2	5
Oligochaeta	Head on	2	4	0
	Side on	0	6	6
Bivalvia		0	2	8
Nematoda	Head on	1	4	3
	Side on	0	3	4
Copepoda	Head on	2	3	0
	Side on	2	5	3
Neochilda fusca	Head on	2	4	0
	Side on	1	3	1
Archiloa wilsoni	Head on	3	4	0
	Side on	0	5	1
Small Turbellaria	Head on	3	4	4
	Side on	0	5	6

See text for more complete descriptions encounter type and response categories.

draws or backs away rapidly in the case of the non-tube dwellers. This response does not include movement of more than one body length of the animal. (2) Move away—the animal may draw back momentarily, but then quickly moves off in a direction away from the turbellarian it encountered. (3) Move forward—the animal moves forward, directly into the turbellarian it has just encountered, forcing it to alter its path. (4) No movement—the animal simply stops all forward motion and remains still.

The spionids responded to both turbellarians in the same way, either withdrawing into the tube and then just remaining there for several minutes afterwards, or by moving out of the tube and away from the turbellarian. Moving out of the tube twice resulted in the spionid being chased and eaten by *Neochildia*. *Archiloa* on two occasions went down into a spionid tube after the animal withdrew. In neither case, however, did *Archiloa* attack the spionid. This does not, however imply that *Archiloa* could not attack and eat a spionid in its tube.

Terebellids also responded in one of two ways. If the encounter was with one or several of the terebellid's tentacles, it would draw in the tentacles and back away. However if the encounter was with the very base of the tentacle or with the head of the animal, it immediately and violently moved forward, thrashing its tentacles and whiplashing its body. This response quickly moved the animal away from the turbellarian, and made it very difficult to chase and capture.

TABLE VIII

Responses of temporary meiofauna to encounter with Neochildia fusca *and* Archiloa wilsoni

		Neochildia fusca				*Archiloa wilsoni*			
Macrofaunal taxa	Mobility	With-draw	Move away	Move forward	No movement	With-draw	Move away	Move forward	No movement
Streblospio benedicti	Occasionally mobile	12	7	0	0	11	5	0	1
Polydora spp.	Occasionally mobile	10	8	0	0	9	6	0	0
Capitellidae	Mobile	12	7	0	0	7	6	0	0
Cirratullidae	Occasionally mobile	9	6	0	0	7	5	0	0
Orbiniidae	Occasionally mobile	4	9	0	0	3	8	0	0
Clymenella torquata	Sedentary	8	2	0	2	4	3	0	4
Axiothella mucosa	Sedentary	2	9	0	0	3	8	0	0
Terebellidae	Sedentary	10	0	11	0	9	0	13	0
Nereidae	Mobile	0	4	9	7	0	4	6	7
Syllidae	Mobile	0	5	6	8	0	3	4	5
Glycera sp.	Mobile	0	1	7	4	0	0	5	6
Arabellidae	Mobile	1	2	0	6	1	0	1	5
Oligochaeta	Mobile	0	13	0	4	0	10	0	0
Bivalvia	Occasionally mobile	Shell closure (6)				Shell closure (7)			
Unmetamorphosed spionid larvae	Mobile	Balls up, spines protruding				Balls up, spines protruding			

See text for more detailed descriptions of categories.

Cirratulids responded, like spionids, by either withdrawing (though they did not build tubes, so they just moved back) or moving away. On one occasion each with both *Neochildia* and *Archiloa,* the animal autonomized part of its gills and left them behind.

Among the four mobile, predatory taxa (syllids, nereids, *Glycera,* and arabellids), withdrawal was almost never a response. *Glycera* on two occasions everted its pharynx upon encountering *Neochildia,* although on neither occasion did it successfully hit the turbellarian. Three nereids also everted their jaws (at 2 *Neochildia,* 1 *Archiloa*) although they also did not actually catch the turbellarian.

On all occasions, bivalves responded to turbellarians by drawing in the foot and closing up the shell. The bivalve usually remained closed for several (2–5) minutes after the encounter.

Observations of undisturbed sediment cores

These observations showed that most of the permanent meiofauna spend very little time exposed on the sediment surface. Animals frequently moved just under the uppermost layer of sand grains. In over 30 hours of observations, nematodes were never seen on the surface for more than a few seconds. They would break the sediment surface, then quickly burrow back down again. Copepods, turbellarians, and ostracods were more active on the surface, but still spent the majority of their time in the sediment. Copepod and ostracod movements were responsible for stirring up and moving around fecal pellets and sediment grains.

Both *Neochildia* and *Archiloa* frequently came to the surface, would move along the surface for a short while (30 s to 5 min), then burrowed back down underneath the sediment grains again. Only one attack on the surface was witnessed. This was a *Neochildia* which swam (flat) into another small turbellarian (*Convoluta* sp.), pinned it to a sediment grain, and ate it. Right after ingestion, *Neochildia* moved below the sediment surface again.

Spionids were active in the cores. A few juvenile *Streblospio* were observed feeding, usually with the tentacles up in the water column. Copepods sometimes swam into the base of a *Streblospio* tube, which resulted in the spionid's withdrawal response in three out of five encounters. A large epibenthic copepod simply swam up into the water column and swam over *Streblospio*'s tentacle field. One *Archiloa* was observed swimming around a *Streblospio* tube, after which it burrowed down between the sediment grains near the tube.

DISCUSSION

Feeding experiments

In interpreting feeding experiments of this type, it is important to remember that what an animal does in the laboratory is not always the same as what it does in nature. These experiments offered the turbellarians only one potential food source at a time. In nature, they, of course, would have a whole range of food sources to choose from. Nevertheless, these experiments do adequately test the simple capability and/or inclination of the predators to eat the various prey offered to them and indicate the general breadth of their diets.

Clearly *Neochildia fusca* and *Archiloa wilsoni* share a number of prey species in common. However it is difficult to make comparisons between species on relative preference for a particular prey species since *Archiloa* was somewhat more reluctant to feed in the laboratory. Very large differences in percentages eaten (Table I) though may in fact be meaningful. *Archiloa* seemed to have a greater preference for copepods, juvenile amphipods, and nematodes than did *Neochildia.* This may have something to do with the way each feeds. A copepod or amphipod, with its unmalleable carapace may be difficult for *Neochildia* to stuff into its mouth. Nematodes may be difficult for *Neochildia* to pin down and/or position under its mouth. Bush (1975), however, reports finding copepod and nematode pieces in the parenchyma of mature *Neochildia.* Thus, either *Neochildia* will occasionally feed on these animals, or these animals were in the gut of another prey species which *Neochildia* ate, like *Archiloa,* a nereid, or a syllid.

It is difficult to know exactly why *Clymenella* was eaten by both turbellarians, and the confamilial *Axiothella* was not (Table I). *Clymenella* has a pelagic larval stage, while *Axiothella* has benthic larvae which develop in a mucus egg case (Newell, 1951). *Axiothella* is not available to the turbellarians until it leaves the egg case. Both *Neochildia* and *Archiloa* seem to be incapable of penetrating the egg case. The juvenile *Axiothella* offered were those which had just left the decomposing egg case, and perhaps had a bit of the mucus still clinging to their bodies. Maybe this mucus prevents their being recognized as prey upon encounter. The replication of these trials was not as large as with some others, so the possibility remains that these results are artificial. The behavioral observations (Tables IV, V) also show an attack on *Clymenella* and not *Axiothella,* but generally the turbellarians withdrew from, or ignored both maldanids.

Archiloa was much more likely to consume only part of its prey than was *Neochildia.* Again this may largely be due to differences in feeding mechanisms. *Neochildia* consumed its prey with extraordinary speed and its central parenchyma is extremely extensible. There is very little time for the prey to escape. *Archiloa* takes much longer to suck out or consume its prey, and is less effective in pinning its prey down, affording the prey greater opportunity for escape. Also, *Archiloa* is not capable of entirely consuming a prey individual much larger than itself (as a >1 mm oligochaete is). A portion of these larger prey appears to be enough to completely fill the gut.

It has been suggested that patterns of turbellarian feeding are directly related to the type of pharynx that each turbellarian possesses (Meixner, 1938; Hyman, 1951; Bilio, 1967; Straarup, 1970). Turbellarians with simple pharynges or lacking pharynges must engulf their prey whole, and thus are limited in the size of prey that they can ingest. Those with plicate or rosulate pharynges which are eversible are able to suck out larger prey. The results of these experiments tend to support this idea. *Archiloa,* with its plicate pharynx, was able to feed on some large prey (*i.e.,* large oligochaetes and nereids) which seemed to be unavailable to *Neochildia* which does not have a pharynx (Table I).

The difference in response of *Neochildia* and *Archiloa* to wounded prey was striking (Table III). Other proseriates have been found in the field clumped around dead animals (Bush, 1966) or have been collected using freshly killed meat as bait (Bush, 1966; Riser, 1981). Vernberg and Coull (1981) reported proseriates swarming around and rapidly consuming recently killed copepods, and suggested that these flatworms had chemotactic abilities. Perhaps the proseriates as a group are able to biochemically detect the presence of wounded animals, by diffusing body fluids, and follow the gradient in these fluids to their source. Also relying on baited trap evidence, Gerlach (1977) suggested that nematodes could do this. If *Archiloa* is at least partially a scavenger as much as a true predator, this would make available to it a food resource which *Neochildia* does not appear to utilize.

Clearly the temporary meiofauna are potentially an important component of both these turbellarian's diets, however the temporary meiofauna are not always abundant. During the times of the year when young macrofauna are not settling in high densities, perhaps food does become limiting. The seasonal influx of large numbers of juvenile polychaetes may allow turbellarian densities to increase to a level not otherwise possible, and may give some of the other permanent meiofauna, such as the copepods, some relief from intense predation, and the opportunity for population density increase as well.

Behavioral observations

Neither observational method, from above or below, proved to be superior to the other. In both cases, if sediment is present in the dish, as it must be for observations to even approach natural conditions, animals are lost from view as soon as they move under or over a single layer of sand grains. This is the major stumbling block to good observations of meiofauna behavior. Even when the animals are visible, they will only be doing something of interest to the observer (like encountering each other) in a fraction of that time. I chose to do more observations from above because the dissecting microscope, with its zoom lens, made it possible to follow an animal longer when it did appear (by zooming down to lower power if it was moving out of the field of view too quickly), or to see something in more detail as it was happening (by zooming up to a higher power). Switching lenses on the inverted scope to do this often resulted in losing the animal or missing the action. Thus perhaps the most important criterion is not viewing angle, but the lens capabilities of the microscope.

Because time was taken to observe these animals in the sediments, most behaviors observed probably do approach natural responses. Mobile taxa moved around, tube-dwellers did build tubes. The angle of the tubes was probably more horizontal than it would have been in the field and appeared in the undisturbed cores, and it is possible there is some artifact associated with this. Juvenile terebellids did not construct well defined tubes or burrows, but only covered themselves with a few sediment grains and detritus particles and laid on the surface of the sediments. This may be normal behavior for juvenile terebellids. Hunt (1981) speculated that juvenile terebellids may be relatively immobile as soon as they settle, but did not know whether they constructed burrows immediately. A juvenile terebellid has only a few tentacles and very incomplete segmentation. The whiplash/thrashing response is a good indication that the musculature is already fairly well developed, but most polychaetes rely on complete intersegmental walls and a complete coelom to burrow efficiently (Trueman and Ansell, 1969). Juveniles may not yet be capable of effective burrowing. The whiplash response may be a uniquely juvenile response, which effectively protects them from small predators like turbellarians, until they are able to burrow into the sediments, and can respond by withdrawal, or they are simply too big to be eaten by these predators.

Many side encounters of *Neochildia* and *Archiloa* occurred with *Streblospio* (Tables IV, V). In dishes of sediment, *Streblospio* builds a tube which may be shorter than it is in nature. When *Streblospio* is withdrawn in its tube, often its posterior end would be out of the end of the tube, and attack would occur on this portion of the animal. Of course *Streblospio* had no tube when dishes of animals were first established, and during this time, animals encountered on the side were very frequently attacked and eaten. Extrapolating to nature then, *Streblospio* is probably most vulnerable right after settlement, but before it has constructed a complete tube. Once the tube is made, the animal is afforded some protection, but is still vulnerable to (less frequent) head on attack when extended out of the tube, or attack from behind when the animal is down in the base of its tube. Since it is nearly impossible to observe animals in the base of their tubes in the natural bottom (or in undisturbed cores), I do not know how common the latter may be. Certainly as the animal grows larger, and its tube extends below the redox layer where these turbellarians are not found, attack from behind would not occur. Also the natural sediments tend to be more consolidated than sediments in a Petri dish, and penetration of the spionid beyond the end of the tube is probably more difficult and less common.

All the animals observed responded much more abruptly to head on encounters than to side on encounters (Tables IV–VII). For the predators (*Neochildia* and *Archiloa*), the overwhelming majority of attacks on prey came from the side rather than from the front. Most of the sensory apparatus is located on the anterior of these animals. Perhaps the response of the prey is important in the decision to attack. In head on encounter, the potential prey has also just sensed the presence of the predator, and is in equally good position to react in a way which might injure the predator. In side encounter, the prey may not be "aware" of what has just touched its side (predator or benign species), or even that something has encountered it, and may make no attempt to move away.

The temporary meiofauna can be divided into two groups based on their responses to encounters with *Neochildia* and *Archiloa*. The first group responded by withdrawal or movement away from the turbellarians (spionids, capitellids, orbiniids, cirratulids, maldanids, oligochaetes), whereas the second group either did not respond at all, or continued moving forward, forcing the turbellarian out of its way (syllids, nereids, glycerids, arabellids) (Table VIII). The first group consists of non-aggressive, largely deposit feeding taxa (Fauchald and Jumars, 1979; pers. obs.). Despite the ability of some spionids to interact aggressively intrafamilially (Levin, 1981), spionids and indeed all the taxa in group one probably have little defense against a predator except moving away. The taxa in group two are all themselves predatory (Fauchald and Jumars, 1979) and aggressive. They could defend themselves against, and even prey upon these turbellarians, although I never witnessed such predation.

Avoidance reactions, particularly the movement away of the taxa in group one above, and the unique thrashing movement of terebellid juveniles are, of course, adaptive, since both *Neochildia* and *Archiloa* can prey on these animals. However, independent of such predation, these reactions could result in negative correlations between densities of both *Neochildia* and *Archiloa* and these taxa in the natural environment. Data from other field experiments (Watzin, 1983), show that experimental increases in turbellarian density did result in reduced densities of some of these groups. Both predation and behavioral avoidance probably contributed to this result. A survey of benthic habitats generally shows much higher densities of turbellarians in sands than in muds (McIntyre, 1969). The opposite trend tends to hold for the polychaetes, especially some of the opportunistic groups such as spionids and capitellids (Sanders, 1958; Commito, 1976). While it may be argued that this distribution pattern is, in part, due to higher organic carbon concentrations in muds (more food), or substrate stability requirements of polychaetes, it may also be due, in part, to the higher densities of turbellarian predators in sandy habitats which may be discouraging settlement of polychaete juveniles, and reducing densities of those that do settle by predation.

There were trends in the responses of juvenile macrofauna to encounter with copepods. *Streblospio*, capitellids, oligochaetes, and *Axiothella* all responded to encounter with *Enhydrosoma* by moving away. Particularly the circling response of *Enhydrosoma* induced these animals to move away. This, combined with my observations and those of others (Cullen, 1973; Rhoads *et al.*, 1977), of the ability of copepods to disrupt the sediment surface which may make it difficult for these animals to construct and maintain tubes and burrows, suggests a negative effect of copepods on these taxa. Again, other experiments (Watzin, 1983) show reduced densities of some of these groups in the field in the presence of high densities of copepods and nematodes.

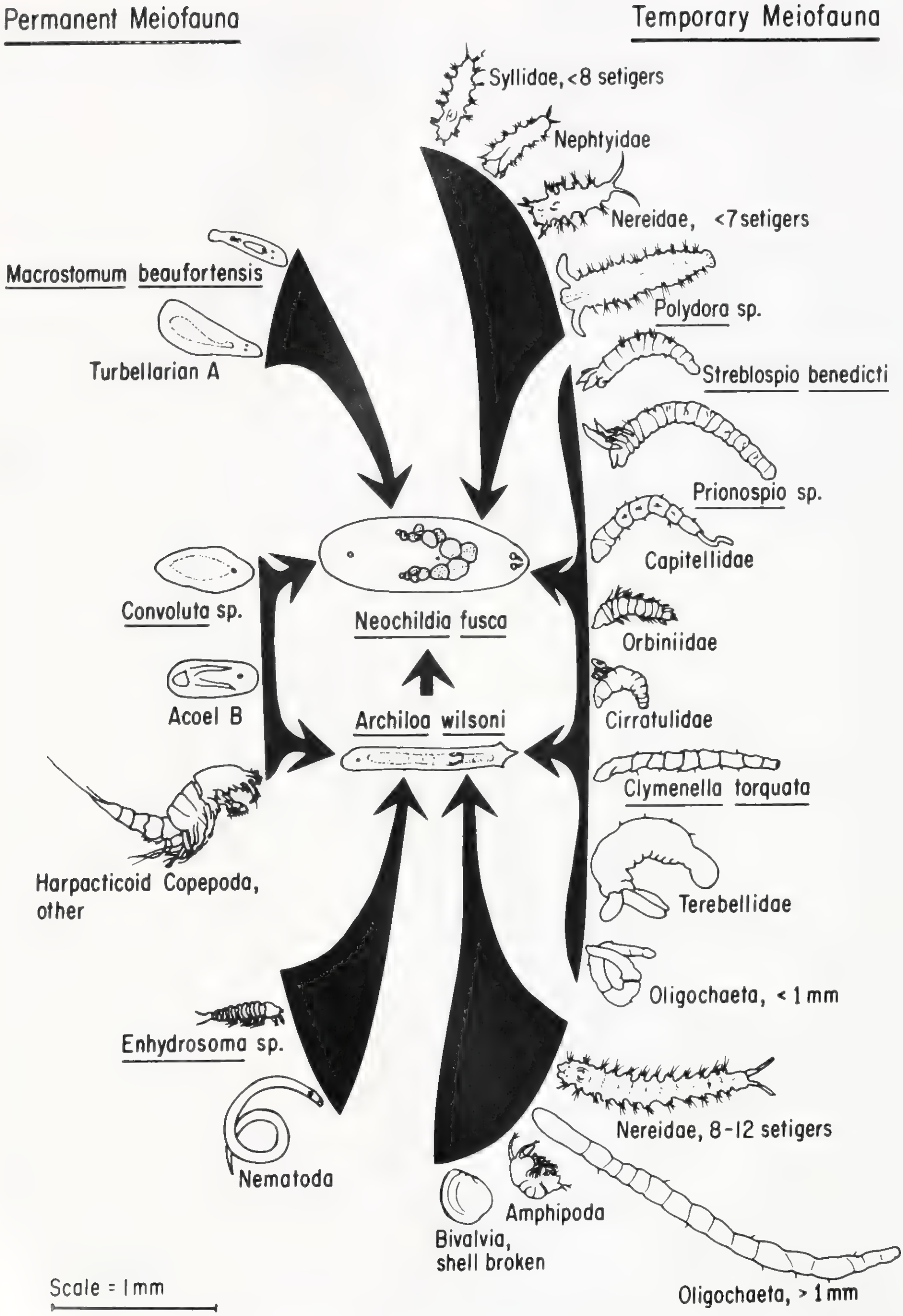
Permanent Meiofauna
Temporary Meiofauna
Syllidae, <8 setigers
Nephtyidae
Nereidae, <7 setigers
Macrostomum beaufortensis
Polydora sp.
Turbellarian A
Streblospio benedicti
Prionospio sp.
Capitellidae
Convoluta sp.
Neochildia fusca
Orbiniidae
Acoel B
Archiloa wilsoni
Cirratulidae
Clymenella torquata
Harpacticoid Copepoda, other
Terebellidae
Oligochaeta, < 1 mm
Enhydrosoma sp.
Nereidae, 8-12 setigers
Nematoda
Amphipoda
Bivalvia, shell broken
Scale = 1 mm
Oligochaeta, > 1 mm

FIGURE 1. A potential food web among the permanent and temporary meiofauna. All animals were drawn to the same scale using a camera lucida.

This paper represents a first attempt to directly observe and interpret the "black-box" of meiofaunal trophic and behavioral relationships. All meiofauna are not simply "packagers" of microbial biomass (Coull and Bell, 1979), but occupy several trophic levels even before passage to epibenthic predators or deposit feeders such as fish (Bregnballe, 1961; Feller and Kaczynski, 1975) or shrimp (Bell and Coull, 1978). Previous attempts have been made at constructing benthic food webs which include the meiofauna, but they have focused on the pathways between micro-organisms and meiofauna (Fenchel, 1970; Coull, 1973), or between permanent meiofauna and macrofaunal consumers (Ankar, 1977; Elmgren, 1978). Based upon the results of these feeding experiments, I have constructed a hypothetical food web among the permanent and temporary meiofauna (Fig. 1).

Turbellarians prey on a wide variety of other meiofaunal taxa, and may play a role in structuring meiofaunal and macrofaunal communities independent of the structure imposed on these communities by even higher trophic levels. Some nematodes are also probably predaceous, but the appropriate feeding experiments and observations have not yet been done to determine this. Before we can completely understand either what factors and interactions control meiofaunal densities and diversities, or what role the meiofauna play in benthic systems as a whole, the complex of interactions between the meiofauna must be better documented.

ACKNOWLEDGMENTS

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THE ECOLOGY OF FERTILIZATION OF ECHINOID EGGS: THE CONSEQUENCES OF SPERM DILUTION, ADULT AGGREGATION, AND SYNCHRONOUS SPAWNING

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ABSTRACT

Percent fertilization of eggs of the echinoid *Strongylocentrotus droebachiensis* (0. F. Müller) was determined both in laboratory and field experiments. In the laboratory, over 50% of the eggs were fertilized only in relatively dense sperm suspensions ($>10^6$ sperm/l); such suspensions retained their potency for less than 20 minutes. In the field, divers induced individual *S. droebachiensis* to spawn with KCl injections. Along five meter transects running directly downcurrent from spawning males, fixed volumes of seawater presumably containing sperm were drawn into syringes already containing eggs. Within 20 cm of spawning males 60–95% fertilization usually occurred; at distances greater than 20 cm less than 15% of the eggs were fertilized. Higher percentages of eggs were fertilized when current speeds were low (<0.2 m/s); swifter currents quickly diluted sperm so that little fertilization occurred. When several males were induced to spawn synchronously, percent fertilization increased but was generally less than 40% at distances greater than 2 m downstream. These results indicate that production of zygotes could be much less than production of eggs. Life-tables based on estimates of egg production may then be in error, unless adults aggregate and spawn synchronously, countering dilution of sperm by currents.

INTRODUCTION

Successful fertilization is a critical step in the life-history of species, particularly for free-spawning marine invertebrates (Mortensen, 1938; Thorsen, 1946; Chia, 1974). Mortensen (1938) noted that although many asteroids produce a large number of eggs, their rates of juvenile recruitment are typically low. He suggested that this discrepancy exists because the probability of eggs encountering sperm in the plankton is low, and that most eggs are never fertilized. In contrast, Thorson (1946) suggested that most eggs of benthic invertebrates are fertilized because adults of both sexes spawn nearly synchronously in aggregations, therefore insuring that sperm and egg encounters occur. In Thorson's (1946) view, low recruitment rates are largely due to predation upon embryos and larvae. Though Thorson's (1946) suggestion has since become more widely accepted, both ideas remain tenable because benthic invertebrates have rarely been observed spawning in the field, and because to date no study has determined field rates of fertilization for any free-spawning invertebrate.

I have thus examined conditions under which spawnings may, or many not, produce fertilized eggs of the sea urchin, *Strongylocentrotus droebachiensis* (O. F. Müller). First, laboratory experiments were conducted to examine the effects of sperm dilution and gamete age on percent fertilization (see Lillie, 1915, 1919; Cohn, 1918; Gray, 1928). Field experiments were then conducted to determine if adult aggregation and epidemic spawning might serve as effective counters to dilution of gametes by tidal

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currents and the effects of gamete aging. The echinoid *S. droebachiensis* was used in this study because: (1) it is a free-spawner, shedding its gametes directly into the sea; (2) adults are motile and can alter their proximity to one another; and (3) the prominent fertilization envelope, which quickly rises from eggs following fertilization (see Tyler and Tyler, 1966), is a convenient indicator of fertilization.

MATERIALS AND METHODS

Laboratory experiments

The first series of laboratory experiments determined over what range of sperm concentration high percentages of fertilization occur. Gametes were obtained by intracoelomic injections of .55 *M* KCl. Two ml of freshly spawned but settled eggs were pipetted into each of a series of 10 jars containing 3 l of filtered seawater, resulting in a concentration of about 3×10^4 eggs per liter. The water in each jar was thoroughly stirred with a paddle which oscillated at about 20 cycles per minute (Strathmann, 1971). Freshly spawned undiluted semen, or "dry sperm," was quickly run through a series of 10, 10-fold dilutions, thus diluting the dry sperm by an order of magnitude at each step. One ml of each dilution was then added to a single jar of the series; the egg/sperm mixtures were allowed to incubate for 10 minutes before eggs were siphoned off, concentrated and fixed in 2% formalin. Assays for percent fertilization were made by counting the number of elevated fertilization envelopes on the first 100 eggs encountered under 160× magnification. Four replicates of this experiment were conducted. Absolute sperm concentrations were assessed by hemocytometer counts of sperm in water from each of the jars.

A second series of laboratory experiments determined the effects of gamete age after spawning on percent fertilization. In experiments to examine sperm longevity, dry sperm was quickly run through 4, 10-fold dilutions. One ml of the most dilute suspension was then added to each of a series of 7 dishes containing 50 ml of filtered seawater at time 0. One ml of settled eggs was added to the first dish at time 0, and in sequence to the remaining dishes at 10 min intervals thereafter. The eggs added to each dish thus encountered sperm of different ages. Eggs were also added to one jar without sperm to serve as a control against inadvertant contamination of eggs by sperm. The egg/sperm mixtures were allowed to incubate for 10 min before they were fixed, and assays for percent fertilization were performed as above. Three replicate experiments were conducted.

Experiments to examine egg longevity were the converse of those described above for sperm. One ml of settled eggs were added to the dishes at time 0, and fresh sperm dilutions (as above) were added in sequence to each of the 8 dishes at 10 min intervals. The freshly diluted sperm added to each dish thus encountered eggs of different ages. The egg/sperm mixtures were incubated for 10 minutes prior to being fixed and assayed for percent fertilization as above. Three replicate experiments were conducted.

A final laboratory experiment was conducted to determine if high percentages of fertilized eggs would result from induced spawning in an aquarium. A 55 gallon aquarium was filled with filtered seawater and allowed to warm to room temperature (14°C). Sea urchins were injected with KCl solution, and when a male and female began spawning they were strapped, mouth down, to plexiglass plates and rinsed. The plates were then placed 50 cm apart on the bottom of the aquarium. The water within the aquarium was not mixed. Samples of eggs were pipetted from the female's aboral surface at 5 minute intervals during the first 20 minutes after spawning began, and also 20 to 24 hours later. Assays for percent fertilization were performed as described above.

Field experiments

Field experiments examined the effects of adult aggregation, current, and epidemic spawning on percent fertilization. A subtidal valley bordering San Juan Channel, Washington, was located where the tidal current usually flows in the same direction and where *Strongylocentrotus droebachiensis* is common about 10 m deep. The bottom of the valley consisted primarily of fist-sized cobblestones. A five meter transect was set up along the bottom running directly downcurrent. Current direction was determined by releasing dye, and current speed was estimated by timing dye movement along the transect. Several *S. droebachiensis* were collected from the vicinity and stimulated to spawn with KCl injections *in situ.* In initial trials, when a female began spawning she was moved upstream of other spawning animals and samples of eggs were pulled directly from over the gonopores into each of 12, 60 ml syringes. A spawning male was then placed at the head of the transect, and any other spawning sea urchins were moved downstream. Along the transect at a series of distances from the spawning male, 10 ml of water were pulled into each syringe about 10 cm over the substrate. The first syringe was filled upstream from the male as a control to detect any extraneous sperm, the second was filled directly over the male's gonopores to assess maximal fertilizability of the eggs, and one syringe each was filled at 10, 20, 40, 60, and 80 cm, and 1, 2, 3, 4, and 5 m downstream from the spawning male. Except for the control, the syringes presumably contained both eggs and sperm. The syringes were then taken to a skiff where their contents were fixed after incubating for a total of 10 minutes. Eggs within the syringes were later assayed for percent fertilization as described for the laboratory experiments. In later trials, the number of eggs per syringe was controlled by adding .08 ml of settled eggs (about 1600 eggs) to each syringe in the laboratory about an hour prior to performing the field experiments.

Similar experiments were conducted under a variety of current conditions, ranging from slack tide with little current (0.05 m/s) to swift currents (0.8 m/s) to examine effect of current on percent fertilization. Experiments as above were also conducted which simulated limited epidemic spawnings, using three spawning males at the head of the transect.

Questionnaire

Because only one direct observation of echinoid spawning was found in the literature (Randall *et al.,* 1964), over 100 sets of questionnaires were mailed to individuals and marine stations in North America, asking for information regarding diver observations of echinoid spawnings. Intertidal observations of spawning were not solicited because osmotic or thermal stresses associated with exposure to air may induce abnormal spawnings (Fox, 1924; Harvey, 1956), and it is doubtful that gametes or embryos survive at low tide in the intertidal zone.

RESULTS

Laboratory experiments

In laboratory experiments to examine the effects of sperm concentration on percent fertilization, high percentages of fertilization were achieved only with relatively dense sperm suspensions (Fig. 1). Over 80% of the eggs were usually fertilized in suspensions containing more than 10^6 sperm/l, but percent fertilization rapidly declined in more dilute sperm suspensions until essentially no eggs were fertilized in suspensions con-

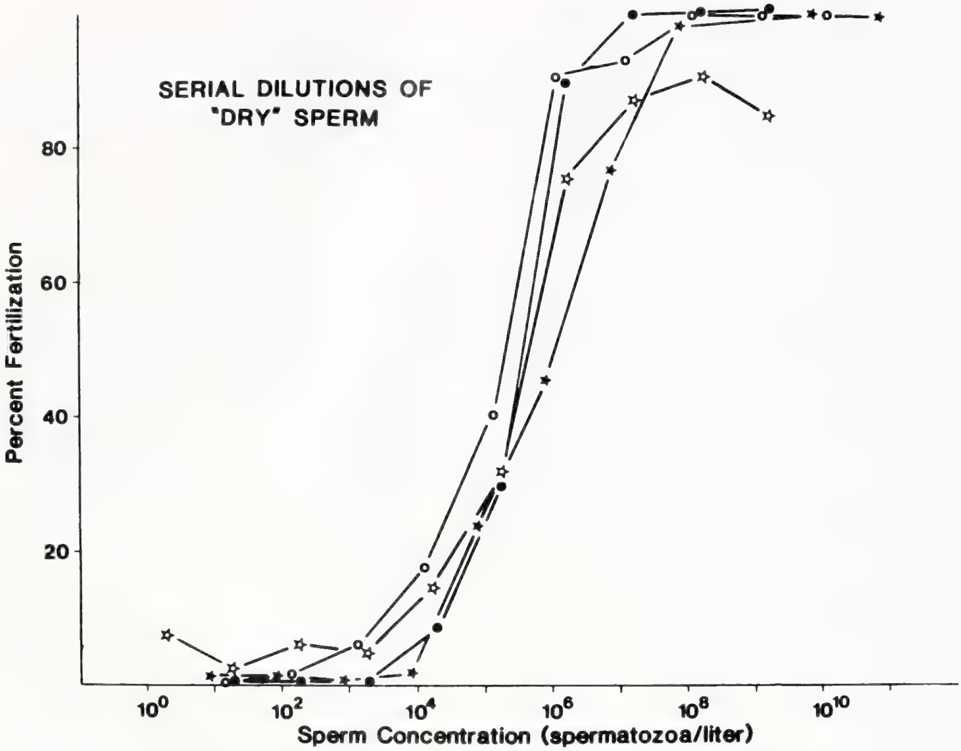

FIGURE 1. Results of four replicate experiments to determine percent fertilization of eggs when constant volumes of a series of 10, 10-fold dilutions of dry sperm (semen) was added to egg suspensions in stirred jars.

taining less than 10^4 sperm/l. Percent fertilization thus rapidly declined when dry sperm was diluted by 6–8 orders of magnitude.

In experiments to examine gamete longevity, diluted sperm suspensions lost potency rapidly so that less than 10% fertilization resulted when eggs were added to 20 min old sperm (Fig. 2A). Eggs, on the other hand, remained fertilizable for at least 90 min after spawning (Fig. 2B).

Results of the experiment to examine fertilization in still water indicated that little fertilization occurred during the first 20 minutes following spawning in extremely still water, but that under these conditions some fertilization continued to occur even 24 hours later (Fig. 3).

Field experiments

Results of the initial series of field experiments show that percent fertilization fell rapidly with increasing distance from spawning males (Fig. 4). Eggs were never fertilized in the control sample taken upstream of the spawning male, whereas over 90% were usually fertilized in the syringe from directly over the spawning male. At distances greater than 10 cm downstream from the male fewer than 25% of the eggs were fertilized, and at distances over 1 m, 10% or fewer of the eggs were fertilized. These low rates of fertilization clearly indicated that the numbers of sperm were limiting, and therefore

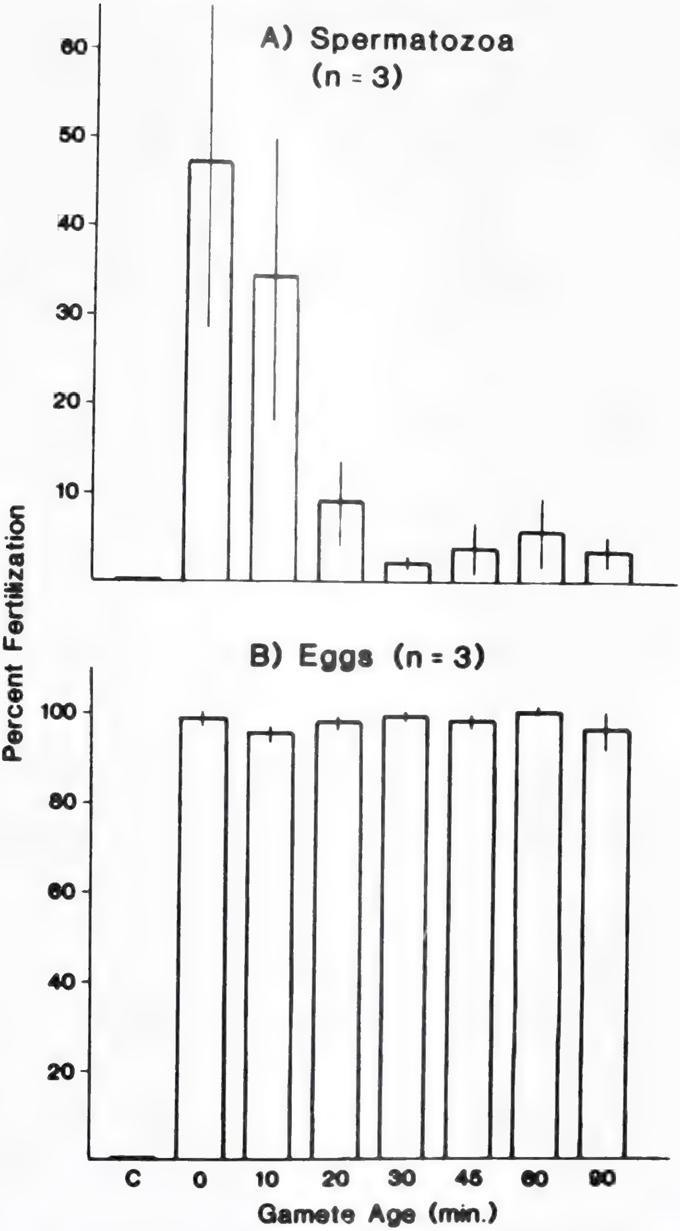

FIGURE 2. Mean percentages of fertilization (±1 standard deviation) resulting from experiments to examine gamete longevity. In A, eggs were added to diluted sperm suspensions of various ages. B is the converse of A, where fresh sperm suspensions were added to eggs of various ages. Three replicates of each experiment were conducted.

that the number of eggs per syringe should be controlled; in subsequent experiments constant numbers of eggs were added to the syringes prior to conducting field experiments. Figure 6A shows that this precaution did not substantially alter the results (for a similar result see Lillie, 1915).

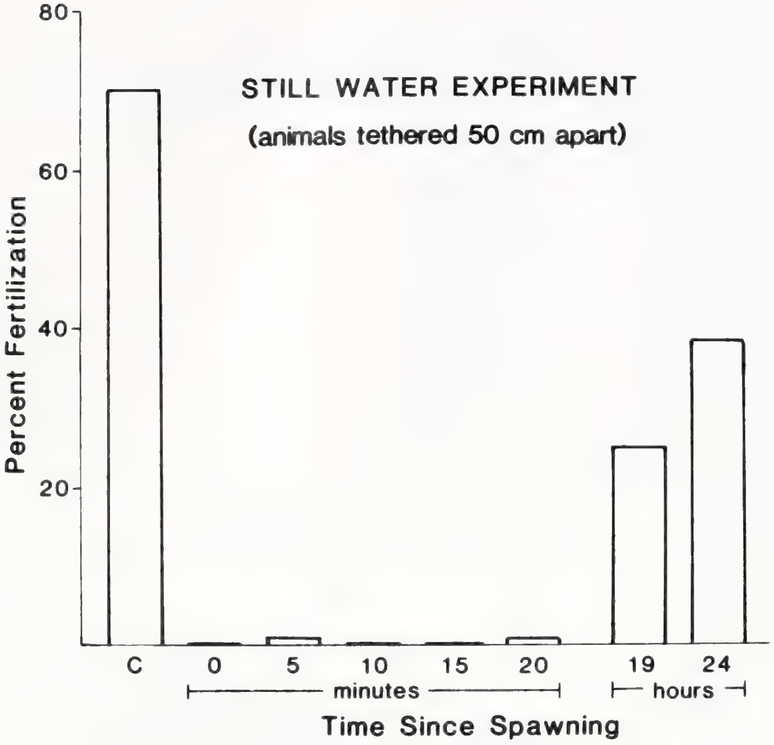

FIGURE 3. Percentages of fertilization resulting from spawning of a pair of animals in still water. A male and female were induced to spawn, strapped mouth down to plexiglass plates, rinsed, and placed in an aquarium 50 cm apart. Eggs were periodically pipetted from the aboral surface of the female and assayed for percent fertilization. At time 0, a control sample of the eggs (C) was fertilized to assess maximal fertilizability.

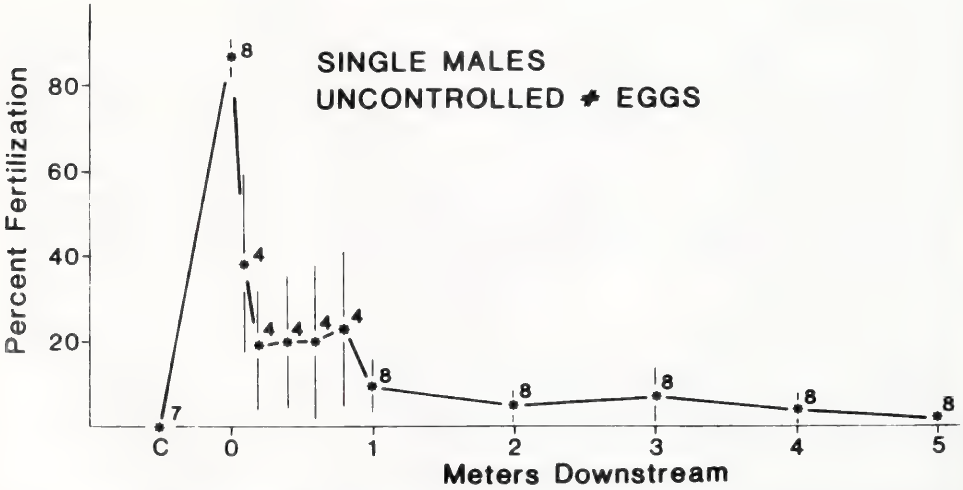

FIGURE 4. Mean percentages of fertilization (±1 standard error) resulting from spawning by single males in the field. Eggs were drawn into the syringes in the field, and 10 ml of water was then pulled into the syringes along a transect running downcurrent from a spawning male. The number above each mean is the number of replicates conducted at that distance from the male.

The effect of current on percent fertilization was examined by conducting experiments in fast or slow-moving water. In fast current (>0.2 m/s; Fig. 5A), percent fertilization was lower at all points downstream than in slower currents (<0.2 m/s; Fig. 5B). However, in both cases percent fertilization declined with increasing distance from the spawning male. At distances over 1 m, percent fertilization was generally less than 20%, even in the slowest currents encountered.

In the simulated epidemic spawnings, percent fertilization was higher at all distances downstream from three spawning males in comparison to experiments where one male was used (Fig. 6A–B). Percent fertilization again decreased with increasing distance from the spawning males, and fewer than 50% of the eggs were fertilized at distances greater than 1 m downstream when three males were used.

Questionnaire

Only seven direct observations of sea urchin spawnings were received in response to the questionnaire, probably reflecting the rarity with which echinoids are observed

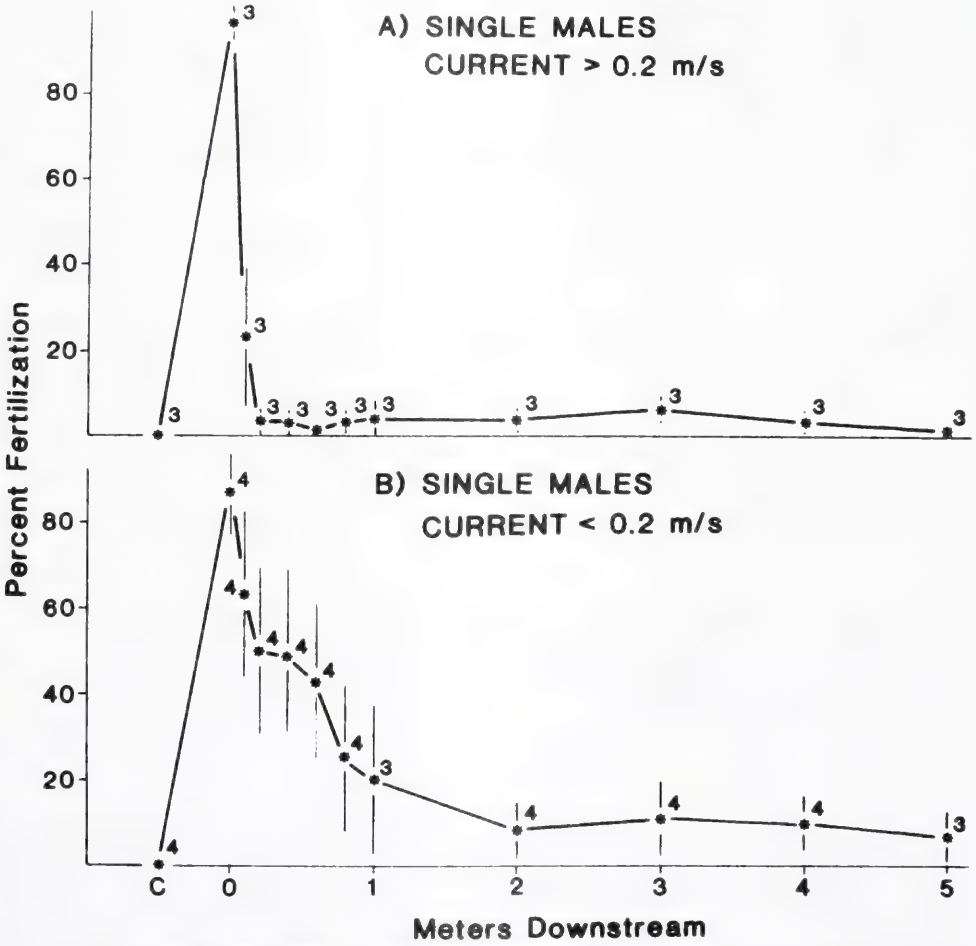

FIGURE 5. Mean percentages of fertilization (±1 standard error) resulting from spawning by single males in currents over 0.2 m/s (A), or under 0.2 m/s (B). Methods were the same as in Figure 4. The number above each mean is the number of replicates conducted at that distance from the male.

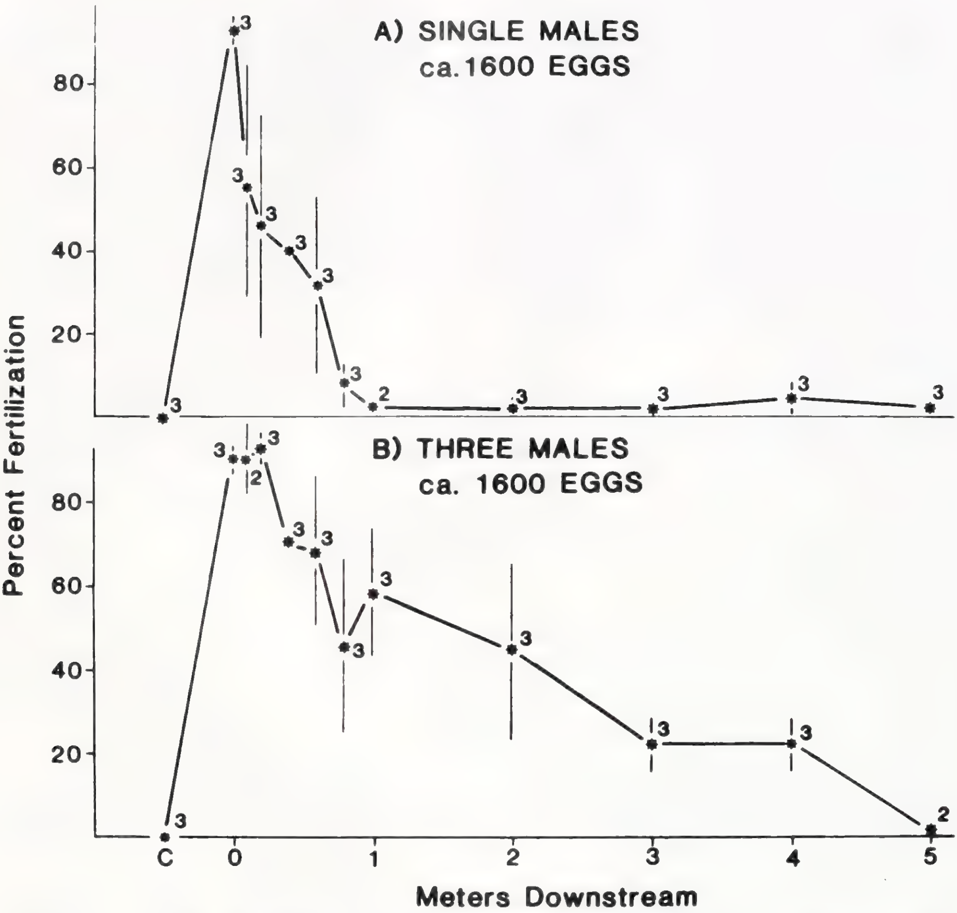

FIGURE 6. Mean percentages of fertilization (±1 standard error) resulting from spawning by single males (A), or three males (B) at the head of the transect. Methods were the same as in Figure 4, except that about 1600 eggs were pipetted into the syringes prior to experiments. The number above each mean is the number of replicates conducted at that distance from the male.

spawning in the field. These observations and the published account (Table I) include four observations of spawning in aggregations and four observations of scattered spawning by a few individuals. These few observations indicate that sea urchins do not always spawn while aggregated.

DISCUSSION

The results of the laboratory experiments indicate that both dilution of sperm and its limited longevity can reduce percentages of fertilization; similar observations have been reported (*e.g.*, Lillie, 1915, 1919; Cohn, 1918; Gray, 1928). It appears that 30–40 sperm for each egg were required to produce high percentages of fertilization (Fig. 1). Several workers have noted similar requirements (Gemmill, 1900; Branham, 1972; Sprung and Bayne, 1984; etc.) which are probably due to the kinetics of random sperm and egg encounters (Rothschild and Swann, 1951). The short potent life of

TABLE I

*Summary of direct observations of subtidal echinoid spawnings**

Species	Season	Density (ind./m^2)	Physical conditions	Spawning behavior	Observer
Diadema antillarum	year-round	1.2–13.4	some current	individuals aggregate year-round; several observations of some individuals within a tight group spawning; upcurrent individuals seemed to spawn first	Randall *et al.*, 1964
Lytechinus pictus	summer	100–500	substantial current; high water temperature	no active aggregation; random individuals spawning; observed several years	R. C. Fay, Pac. Bio-Mar. Labs., Venice, California
Unknown (Apra Harbor, Guam)	spring	75–100	slight current	strongly clumped; mass spawning	G. Pittenger, U.S.C. Mar. Lab., Avalon, California
Heliocidaris erythrogramma	spring	0.1–0.5	no swell or surge; a rare, oily calm	clumped; eggs later seen floating as "rafts"	S. A. Sheperd, Dept. Fisheries, Adelaide, S. Australia
Stronglylocentrotus franciscanus	winter: early spring	unknown	slight current	no active aggregation; scattered individuals spawning	G. Dennis, Comox Diving Services, Comox, British Columbia
Stronglylocentrotus franciscanus	unknown	*ca.* 5	little current or surge	dozens of individuals spawning near other conspecifics crushed by an anchor	J. S. Pearse, UCSC, Santa Cruz, California
Stronglyocentrotus purpuratus	spring	50–60	sea calm; no surge	no active aggregation; scattered groups spawning; crushed *S. franciscanus* nearby	C. T. Mitchell, MBC Applied Env. Sci., Costa Mesa, California
Stronglyocentrotus purpuratus	winter; spring	20–100	moderate surge; low salinity	no active aggregation; random individuals spawning in December and January; mass spawning in April; crushed conspecifics did not induce spawning among intact individuals	R. C. Fay, Pac. Bio-Mar. Labs., Venice, California

* Unpublished *personal communications* were obtained as responses to over 100 sets of questionnaires mailed to individuals and marine stations in North America.

diluted echinoid sperm (Fig. 2) is known as the "respiratory dilution effect" (reviewed by Chia and Bickell, 1983). In essence, sperm in dense suspensions remain quiescent as in the testis, but with dilution the sperm become increasingly active and are rapidly exhausted. Both of these factors restrict the conditions under which field spawnings might produce high percentages of fertilization. Sperm must not only be dense, but must be spawned (or diluted) only minutes prior to encounters with eggs.

In the field, percent fertilization was generally low at distances over 10 cm from spawning males. In laboratory experiments dry sperm diluted by 6–8 orders of magnitude produced similar percentages of fertilization. However, even in the slowest currents encountered, sperm was less than 10 minutes old before it drifted beyond the end of the transect. The limited longevity of diluted sperm thus did not affect results of experiments, and the decreases in percent fertilization observed were probably due to dilution alone. Even if the sperm were long-lived, they would probably be so quickly diluted after spawning that encounters with eggs in the plankton would be rare in any case. The results of all field experiments indicate that if males and females spawn at distances of even a few meters from each other, percentages of fertilization will be very much reduced in comparison to animals that spawn in close proximity.

In all field experiments the syringes contained enough eggs to, in effect, estimate sperm density in the surrounding water. I was thus unable to examine rates of fertilization under conditions where egg suspensions were very dilute. If females spawned some distance upstream from males, possibly inducing downstream males to spawn with a water-born pheromone (see Reese, 1966; Giese and Pearse, 1974), higher sperm-to-egg ratios per unit volume of water downstream from the males might allow higher percentages of fertilization to occur in these areas. However, because echinoid sperm only swim about 2 cm during their potent life (Gemmill, 1900) and do not detect eggs at a distance (reviewed by Rothschild, 1956; Chia and Bickell, 1983), only those eggs that drift directly past spawning males should be fertilized; eggs drifting elsewhere should not encounter sperm.

Percentages of fertilization in slow currents were higher than in swift ones (Fig. 5), presumably due to the increased rate of dilution of sperm in swift-moving water. However, if sea urchins spawn into extremely still water, laboratory results indicate that little fertilization would occur (Fig. 3). Eggs and sperm simply accumulated on the aboral surface of the spawning animals and did not mix, resulting in low rates of fertilization. Because the sperm remained undiluted on the aboral surface of the male, it remained potent and continued to fertilize some eggs for 24 hours after spawning began. These results are almost certainly laboratory artifacts; such extremely still water should rarely, if ever, occur in neritic habitats. In summary, the above results indicate that higher percentages of fertilization in the field will occur if free-spawning animals spawn into quiet, rather than swift-moving water.

When epidemic spawnings were simulated by placing three spawning males at the head of a transect, water downstream presumably contained more sperm per unit volume and thus percentages of fertilization were higher at all distances from the males (Fig. 6). As circumstances that produced high sperm densities also produced the highest percentages of fertilization, percent fertilization would probably be still greater if larger numbers of spawning males had been used.

It therefore appears that adults must first aggregate and then spawn synchronously if high percentages of fertilization are to occur. Mortensen (1938) did not believe that most asteroids exhibit such social behaviors, although at least three asteroid species aggregate or even pair during spawning seasons (Mortensen, 1931; Clemente and Anicete, 1949; Kubo, 1951). In contrast, Thorson (1946) suggested that synchronous spawning of adult aggregations occurs among most species of free-spawning invertebrates. However, Thorson (1946) based his predictions primarily on observations of laboratory spawnings, where a variety of stresses can cause spawning (Fox, 1924; Harvey, 1956) and where aggregation is typically enforced.

Echinoids have often been reported in aggregations during spawning seasons (reviewed by Boolootian, 1966; Reese, 1966), but it has rarely been determined if sea urchins are actually attracted to conspecifics or if they have simply converged upon some common resource such as food, or shelter during daytime (Mortensen, 1943; Moore *et al.,* 1963; Randall *et al.,* 1964; Pearse and Arch, 1969). In the single study where conspecifics have been shown to actively aggregate (Dix, 1969), the behavior was not confined to the spawning season and it was suggested that *Evechinus chloroticus* aggregates not for spawning purposes, but for mutual protection. Tennent (1910) did report that *Lytechinus variegatus* found in groups were often spawned-out, while scattered individuals contained gonads in varying states of maturity. Direct observations of echinoids spawning in the field (Table I) indicate that sea urchins do not always spawn in aggregations. It therefore remains possible that echinoids do not aggregate preparatory to spawning. However, some echinoids do migrate into shallow water during spring and summer, becoming abundant in the shallow subtidal (Elmhirst, 1922; Orton, 1929; Stott, 1931). Echinoids have also been reported to move in feeding "herds" or "fronts" (Foreman, 1977; Mattison *et al.,* 1977; Witman *et al.,* 1982; etc). Regardless of whether these behaviors are truly social interations, higher percentages of fertilization should result if sea urchins spawn during periods of high population density rather than if they spawn while scattered.

Although all experiments simulated synchronous spawnings, the brief potent life of dilute sperm and the absence of fertilization in control syringes filled upstream of spawning males both suggest that no fertilization would occur if spawning was asynchronous. However, the observations in Table I indicate that synchronous local spawning is typical. Presumably, local environmental cues synchronize gametogenic cycles and induce spawning ("proximal causes"; Baker, 1939). In the field proximal cues may be temperature and salinity changes or thresholds, lunar or tidal cycles, changes in quantity or quality of illumination, and increases in phytoplankton or food abundance (reviewed by Giese, 1959; Boolootian, 1966; Giese and Pearse, 1974; Himmelman, 1981; Chia and Bickell, 1983). However, the effects of most proximal cues on spawning remain questionable because portions of a local population commonly become spawned-out weeks before others (MacGinite and MacGinite, 1949), and conversely, because spawnings frequently occur when there has been little or no change in a cue (Giese and Pearse, 1974). Some of the above confusion may arise because it has often been difficult to distinguish between factors that entrain gametogenic cycles and those that initiate spawning. Results of laboratory experiments to examine proximal cues as spawning inducers are difficult to interpret because spawning is often induced by exposing animals to stimuli in quantities that are potentially stressful to adults, gametes, or embryos (Harvey, 1956; see Farmanfarmian and Giese, 1963; Andronikov, 1975; Greenwood and Bennett, 1981).

For echinoderms it is also unclear whether proximal cues might function by inducing entire populations to spawn, or whether they stimulate or stress a few susceptible individuals within a population to spawn. It is widely suggested that once spawning by one or a few animals is initiated, either the gametes or a pheromone released with them induce neighboring conspecifics to spawn (MacGinite and MacGinite, 1949; Hyman, 1955; Rothschild, 1956; Reese, 1966; Giese and Pearse, 1974; Kennedy and Pearse, 1975; Iliffe and Pearse, 1982). Fox (1924) and Lewis (1958) induced sea urchins to spawn by adding sperm suspensions to seawater in the laboratory, and Gemmill (1914, 1920) described similar experiments with starfish. However, many attempts to repeat these experiments have not resulted in spawning (Gemmill, 1900; Palmer, 1937; *pers. obs.*). In the field, Kěckeš *et al.* (1966) induced *Paracentrotus lividus* to spawn by exposure to homogenates of conspecific testes or ovaries, and two of the observations of spawning in Table I were made in the vicinity of sea urchins that had been crushed. In one instance the crushed individuals were conspecific to those that were spawning, but in the second case crushed *Strongylocentrotus franciscanus* may have induced individuals of *S. purpuratus* to spawn. Nevertheless, several of the observations of Table I indicate spawning by only some individuals within a locale.

Though all free-spawning invertebrates encounter the problem of gamete dilution, there is certainly variation in the mechanisms utilized to increase percentages of fertilization. As one example, sperm of some hydromedusae and species of benthic invertebrates chemotactically sense conspecific eggs and swim towards them (Miller, 1979, *in press*). In this case it may be advantageous for sperm to be long-lived or even to remain quiescent until they sense nearby eggs. The evolution of blocks to polyspermy (see Rothschild, 1956) in eggs of free-spawning species clearly indicates that, at least occasionally, eggs encounter sperm in abundance.

In summary, the experiments presented here indicate that if free-spawning adults fail to aggregate prior to spawning, as Mortensen (1938) proposed, percentages of fertilization will often be low. Conversely, if they spawn in aggregations percentages of fertilization will be high, as Thorson (1946) suggested. It remains uncertain as to whether echinoids are gregarious prior to spawning, but it appears that local spawning is often synchronous. If free-spawning invertebrates do not aggregate and then spawn synchronously, life-tables based on estimates of egg production may overestimate fecundity.

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A NEW MODEL OF PODIAL DEPOSIT FEEDING IN THE SAND DOLLAR, *MELLITA QUINQUIESPERFORATA* (LESKE): THE SIEVE HYPOTHESIS CHALLENGED

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ABSTRACT

The feeding mechanism of *Mellita quinquiesperforata* (Leske) has been examined in detail. This sand dollar is a deposit feeder, ingesting particles mostly in the range of 100–250 μm. The particles are picked out of the substrate individually by specialized long barrel-tipped podia, which form a narrow palisade surrounding the geniculate spine fields on the oral surface. Selected food items are passed to short barrel-tipped podia, thence from podium to podium until they reach the food grooves where they are finally aggregated into mucus cords. The cords are passed to the mouth by the activity of food groove podia. At the peristome, the cord is passed between the circumoral spines by large food groove podia and steered into the mouth by five pairs of buccal podia. The lantern is powerfully muscled and has hardened teeth which crush diatoms and fracture many sand grains. For this reason, there is an apparent accumulation of fine particles (<50 μm) in the gut. Analysis of size frequencies of the material in the mucus cords and substrate indicates that no selection of fine particles occurs and, in fact, that they are virtually absent from the native sediment. An account of spine and podial morphology and distribution is included with descriptions and measurements of surface ciliary currents. It is shown that the formerly accepted sieve hypothesis of feeding cannot be entirely rejected on theoretical grounds. However, during feeding there was no evidence of the operation of any of the elements of the supposed sieve mechanism. Furthermore, the ciliary currents are not fast enough to account for the movement of most ingested material. Patterns of ciliary flow on the oral surface are not simply centripetal, but are much more complex than previously supposed.

INTRODUCTION

The precise nature of the feeding mechanism in sand dollars has become a matter of some controversy. Two quite different mechanisms have been proposed. The first postulates that the aboral surface spines act as a sieve which selects small particles. The second postulates that podia of the oral surface are the primary food collecting organs. The sieve hypothesis originated in an account of *Dendraster excentricus* (Eschscholtz) by the McGinities (1949) and was presented, more or less in its present form, by Goodbody (1960) following a study of *Mellita (Leodia) sexiesperforata* (Leske). According to this hypothesis, fine organic and inorganic particles drop between the spines on the aboral surface. The particles are picked up in ciliary currents and swept around the ambitus (or via the lunules) to the oral surface, where they eventually enter the food grooves. Recent supporters of this hypothesis [Chia, 1969 for *D. excentricus;* Lane, 1977; Ghiold, 1979, Alexander and Ghiold, 1980, Lane and Lawrence, 1982,

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Smith and Ghiold, 1982 for *M. quinquiesperforata;* Mooi and Telford, 1982, Ghiold, 1983 for *Echinarachnius parma* (Lamarck)] allowed, at most, a secondary role for podia in particle collection although they recognized a role in food transport. The podial mechanism for clypeasteroid feeding was espoused by Nichols (1959) and later by Telford *et al.* (1983) for *Echinocyamus pusillus* (O. F. Müller); by Ellers and Telford (1984) for *Echinarachnius parma;* some elements of it were described for *D. excentricus* by Timko (1976), and it is implied, but unstated, as a generality by Kier (1974). Mooi (1983, 1985a, b) has described an array of morphological differences in podia and podial distributions which can best be explained in light of the podial feeding hypothesis, although he and Telford (1982) had once inclined to the sieving hypothesis. According to the podial hypothesis, food particles such as diatoms, organic debris, or grains of sand laden with nutrients, are individually picked out of the substrate by podia and then passed from one podium to the next until they reach the food grooves.

It is a central tennet of the sieve hypothesis that the particles must be small (<100 μm) in order to move through the interspine spaces and to be transported by ciliary currents. Major criticisms of the hypothesis (Ellers and Telford, 1984; Telford, 1983) have focused on the following points: (i) inclusion in the gut contents and the food cords of numerous particles too large to be accommodated by the sieve mechanism; (ii) unsuitability of the velocity and direction of ciliary currents as transport vehicles, and (iii) lack of direct observations and experimental data that provide adequate support. These criticisms have been based primarily on experience with the nonlunulate species, *E. parma,* and on evaluation of the published findings of other investigators. Most of the proponents of the sieve hypothesis have worked with lunulate sand dollars, most notably with *M. quinquiesperforata,* which might be quite different in their modes of feeding.

In this paper we provide a completely new description of anatomy and feeding in *Mellita quinquiesperforata,* including data on: spine types; ciliary currents; diversity, structure, and function of podia; and collection, transport, and ingestion of food. These observations show that podial selection is, indeed, the principal feeding mechanism. In addition, we will show how confusion with ciliary-borne material might have arisen.

MATERIALS AND METHODS

Specimens of *M. quinquiesperforata* were collected at Bird Shoal (Beaufort, North Carolina) in March 1984, and maintained in running seawater and natural substrate in the laboratory. Specimens ranging from 2 mm to 75 mm in diameter were used in this study. Some specimens were fixed in the field, immediately after collection. For SEM examination the fixative was 2% glutaraldehyde. For general histology and examination of gut contents, 10% buffered formalin in seawater was used. After fixation, specimens were stored in 3% neutral buffered formalin. Ten substrate samples, 20 mm deep and 50 mm in diameter, were collected from sites beside individual sand dollars and fixed in 10% buffered formalin to preserve living organisms and organic material. Larger samples were taken and kept fresh for use in the holding tanks and for feeding observations. Abundant supplies of diatoms were obtained in a plankton net towed in well-mixed, sediment-laden water near Bird Shoal.

Podia were classified according to the types described by Mooi (1983; 1985a, b) and their distributions on the sand dollars were mapped. Similar maps of the distribution of the different types of spine were prepared. Spines were measured by ocular micrometer; inter-spine and inter-podial distances were estimated from live and freshly killed specimens. Distribution of cilia on different spine types was examined by light microscopy of isolated spines. Measurements of podia were made on live specimens, using an ocular micrometer, and supplemental measurements of tip dimensions were made from SEM micrographs of critical point dried material (Mooi, 1983).

Observation of live specimens in glass aquaria were made mostly with a stereoscopic microscope. The aboral surface could readily be viewed from above; the oral surface was examined with a horizontally mounted microscope and an inclined, front-silvered mirror or directly, using an inverted compound microscope. The same horizontal microscope was also suitable for study of the ambitus. Cold, fiber optic light sources were used for all observations. These methods were satisfactory for preparing detailed maps of ciliary currents, observation of spine and podial movements, as well as tracking individual food particles during feeding. Ciliary currents were made visible by fine streams of fluorescein in sea water, introduced from a microsyringe via a narrow tipped capillary tube. Tracer dyes disperse too rapidly for prolonged observation, for which finely ground carmine particles were used. Feeding was observed with a thin layer of substrate on the aquarium floor and was stimulated by offerings of sand enriched with diatoms obtained from plankton tows. Rates of ciliary currents and movements of particles were determined by stopwatch and ocular micrometer. At any single point, the currents vary considerably from time to time and are difficult to measure precisely. Our current velocity estimates are based on 5 to 10 determinations at each locality, and have an error of plus or minus 20%.

Analyses of natural substrate (10 replicates), gut, and food groove contents (of 5 individuals) were made by counting particles. Very small samples of well-mixed material were strewn on microscope slides, all particles were drawn in outline by camera lucida, at least 500 were then measured and counted from each sample. Acid soluble carbonate was determined gravimetrically following digestion in 50% HCl. Diatoms were identified to genus (Griffith, 1961). For comparative purposes, freshly fractured sand grains were obtained by crushing between two microscope slides.

RESULTS

The following description of spination is from an adult animal. Spines grow with negative allometry, becoming relatively smaller as test diameter increases (Seilacher, 1979). Measurements given in Table I are for an individual, 75 mm in diameter. The aboral surface is covered by club-shaped spines (Fig. 1A) ("shoe" spines of Seilacher, 1979) with their expanded tips directed forwards in the anterior midline and diverging right and left on the sides. Towards the posterior the club spines gradually become oriented at right angles to the anterior-posterior axis of the test. Each club spine is surrounded by five or six miliary spines. The aboral miliary spines (Fig. 1B) are characterized by large sacs at the tips which fill in the spaces between the swollen tips of the club spines. Together, the miliary sacs and club spines form a complete canopy over the aboral surface. Beneath this canopy the spine shafts are approximately 100 μm apart, although the exact distance in live specimens is constantly changing. Sacs were not included in the miliary spine measurements of Table I. Close to the lunules, the club spines show continuous variation in form, merging smoothly into the paddle-shaped lunule margin spines (Fig. 1C). Around the ambitus there is a fringe of larger spines (Fig. 1D). Ambital fringe spines increase slightly in length from anterior to posterior. On the oral surface the principal spine types are locomotory, geniculate, and pressure drainage channel spines. Locomotory spines (Fig. 1E) are arranged in narrow wedges in Lovén's interambulacra 1–4 and as elongate patches posterior to the anal lunule in interambulacrum 5 (Fig. 3A). Anteriorly the locomotory spine patches of interambulacra 2 and 3 are united (Fig. 3A). Locomotory and ambital

TABLE I

Lengths of different spine types (μm) from one specimen of Mellita quinquiesperforata, *75 mm diameter*

Spine type	N	Mean	±S.D.
Anterior fringe	20	2015	254
Posterior fringe	20	2367	352
Locomotory	20	1846	162
Pressure drainage channel (pdc)	25	1537	247
Geniculate	25	574	68
Geniculate (among pdc spines)	13	716	79
Circum-oral	15	1705	165
Circum-anal	16	2270	419
Club	34	690	36
Aboral miliary	32	605	32
Oral miliary	25	733	72
Ant. lunule, fringe	16	1481	285
Ant. lunule, wall	20	1458	225
Ant. lunule, miliary	25	691	68
Anal lunule, fringe	15	1692	243
Anal lunule, wall	20	1234	130
Anal lunule, miliary	24	568	45

fringe spine types are somewhat alike in form and intergraded in size. Areas not covered by locomotory spines are mostly covered by geniculate spines (Fig. 1F). The shaft of geniculate spines is bent near the mid-point. These spines are oriented with their bent tips directed towards the interambulacra (Fig. 4) and pointing into the ciliary current flow (Fig. 5). They do not form interlocking baskets beneath the food grooves. In each ambulacrum there is a shallow depression with lateral tributaries. In ambulacrum III this depression expands gradually until it reaches the ambitus; in the other ambulacra the depressions continue smoothly into the lunules. These were called "food tracts" by Goodbody (1960) and "pressure drainage channels" by Telford (1983). The latter terminology (pdc) will be used throughout this paper. Within the pdc's, the spines (Fig. 1G) are longer and less bent than geniculate spines. They are much more widely spaced, and sparsely interspersed with miliary spines bearing small sacs on their tips. Elsewhere, scattered miliary spines may be encountered. Smaller versions of the pdc spines also occur on the inner walls of the lunules. Surrounding the mouth there are three tiers of straight, stout circum-oral spines (Fig. 1H). Around the anal papilla there is a cluster of longer anal spines (Fig. 1I). All spine types possess lengthwise bands of cilia on opposite sides of the shaft (Figs. 1A–I) and extending to different lengths along it. Ciliary currents flow at right angles to the ciliary bands. The orientation of ciliary bands depends more on the position of the spine on the test than on the shape of the spine itself.

Six morphologically distinct types of podia (Fig. 2A–F) can be recognized: (i) accessory; (ii) long barrel-tipped; (iii) short barrel-tipped; (iv) food groove; (v) large food groove; and (vi) buccal. Each is distributed in a characteristic, unique pattern (Fig. 3B). Accessory podia (Fig. 2A) are found in a discontinuous fringe around the ambitus. They are absent in interambulacra 2 and 3 where the locomotory spine fields extend to the ambitus, and at four isolated patches of locomotory spines anterior to ambulacra II and IV and also where the pressure drainage channel of ambulacrum III intersects the ambitus. The resting podia are shorter than the surrounding spines, but they are capable of extending to 5–6 mm. The podial stem is very long, narrow,

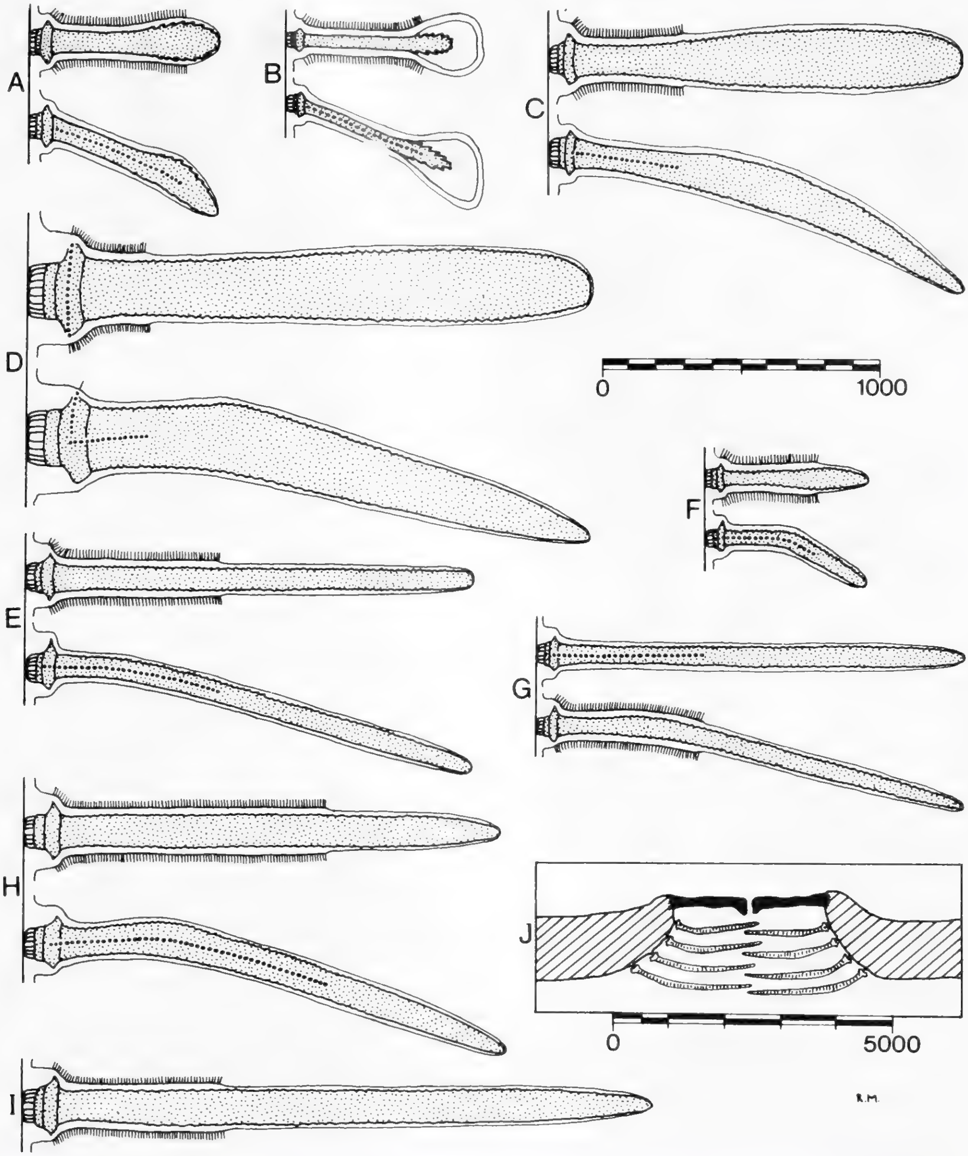

FIGURE 1. Spine types of *Mellita quinquiesperforata*. (A) club-shaped, (B) aboral miliary, (C) lunule margin, (D) fringe, (E) locomotory, (F) geniculate, (G) pressure drainage channel, (H) circum-oral, (I) anal. Spines in A–H are shown in two views, ciliary bands shown edge-on as a row of dots. Inset, J, is a cross-section through the peristome showing tiered arrangement of circum-oral spines. Stereom cross-hatched, peristomial membrane in solid black. All scale bars in μm.

and flexible, terminating in a small, round, suckered tip (up to 100 μm diameter) with sensory cilia. There is only a very narrow band of accessory podia fringing the aboral surface, with a few extending towards the lunules in the ambulacra. On the oral surface, between the locomotory spine fields, there are numerous barrel-tipped (b-t) podia. Barrel-tipped podia (Mooi, 1985b) have cylindrically expanded tips, approximately

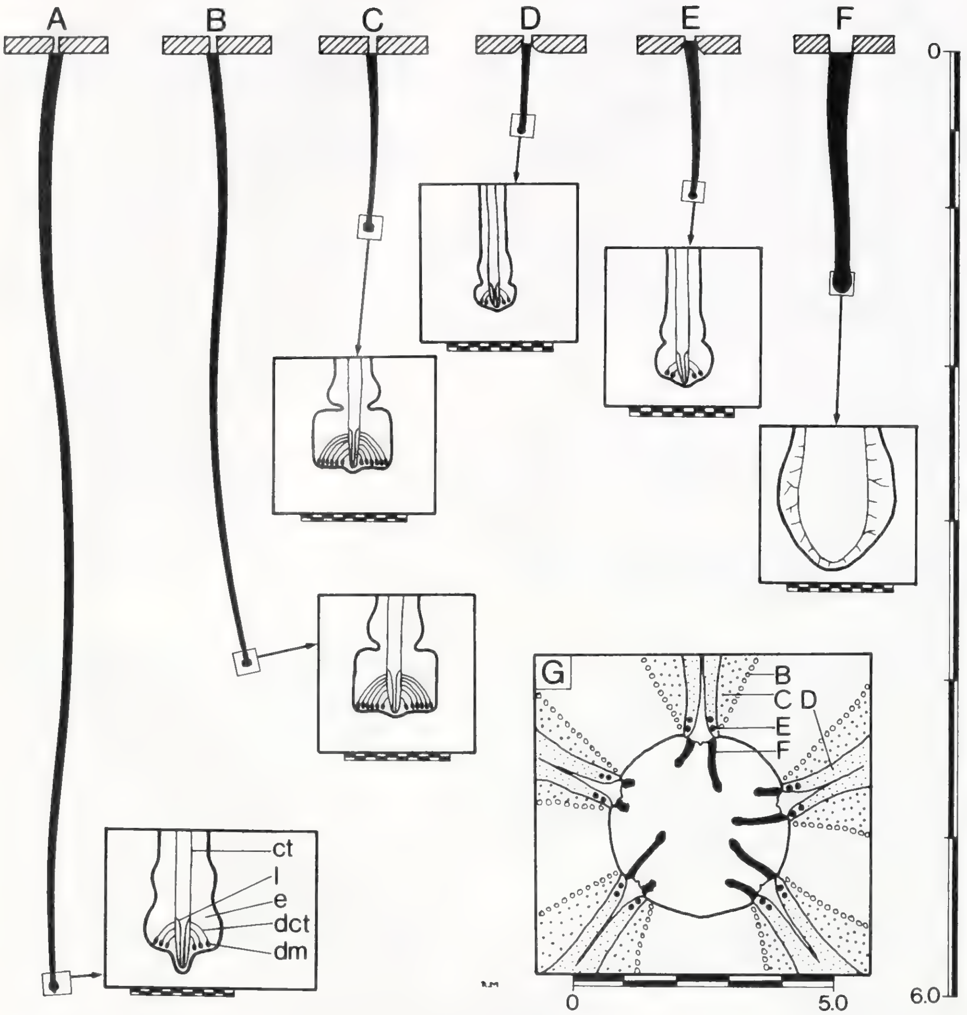

FIGURE 2. Podial types of *Mellita quinquiesperforata*. For A–F, podia in solid black are fully extended, scale bar at right in mm. (A) accessory, (B) long barrel-tipped, (C) short barrel-tipped, (D) food groove, (E) large food groove, (F) buccal. Each podium is accompanied by a close-up of the tip with accompanying scale bar of 100 μm. Structures are as labelled in A: c, connective tissue sheath; e, external epithelium; l, levator muscle; dct, disk connective tissue; dm, disk muscle. (G) shows arrangement of podia around the peristome, scale bar in mm. Letters indicating podial types as in rest of figure.

120 μm in diameter (Fig. 2B, C). In *M. quinquiesperforata* there are two distinct forms, long and short. Long b-t podia are restricted to narrow strips (up to ten podial rows in width) along the edges of the pressure drainage channels and the locomotory spine fields. Thus they surround the geniculate spine zones. These podia can reach beyond the longest spines of the oral surface, a distance of some 3–4 mm. Short b-t podia have exactly the same distribution as the geniculate spines. They are less extensible and seldom reach 1 mm (less than the length of locomotory spines). In some clypeasteroid species there is also a distinction between large and small b-t podia (Mooi,

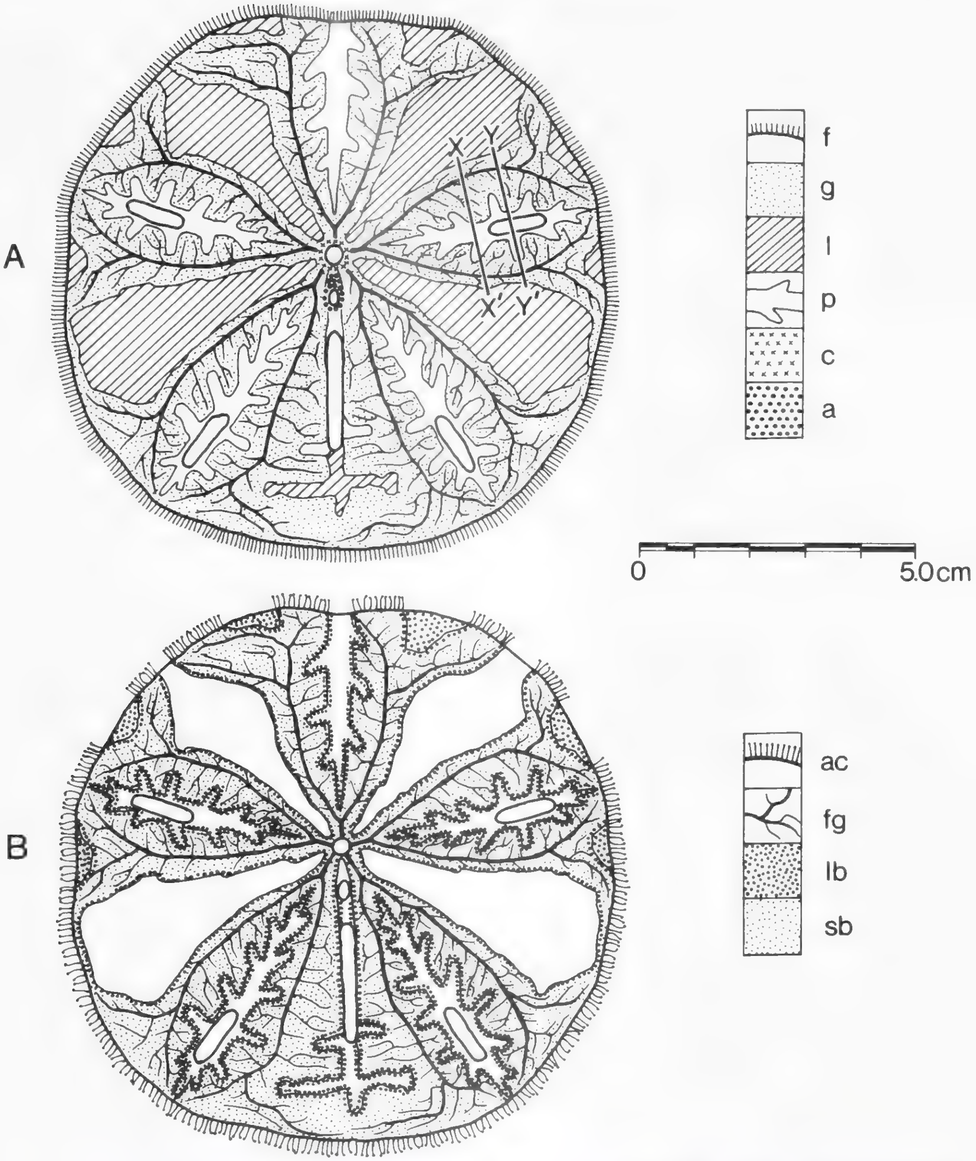

FIGURE 3. Distribution of (A) spines, and (B) podia on oral surface of *Mellita quinquiesperforata.* Transects XX′ and YY′ are shown in Figure 4. Spines: f, fringe; g, geniculate; l, locomotory; p, pressure drainage channel; c, circum-oral; a, anal. Podia: ac, ambital accessory; fg, food groove; lb, long barrel-tipped; sb, short barrel-tipped.

1985b), but this is not clearly apparent in *M. quinquiesperforata.* Other podial types are exactly as described by Mooi (1985a, b). The stubby food groove podia (Fig. 2D) are relatively inextensible, seldom reaching more than 0.5 mm in length and are confined to the walls and ceilings of the food grooves themselves. Close to the mouth large (1 mm) food groove podia (Fig. 2E) occur and, surrounding the mouth (Fig.

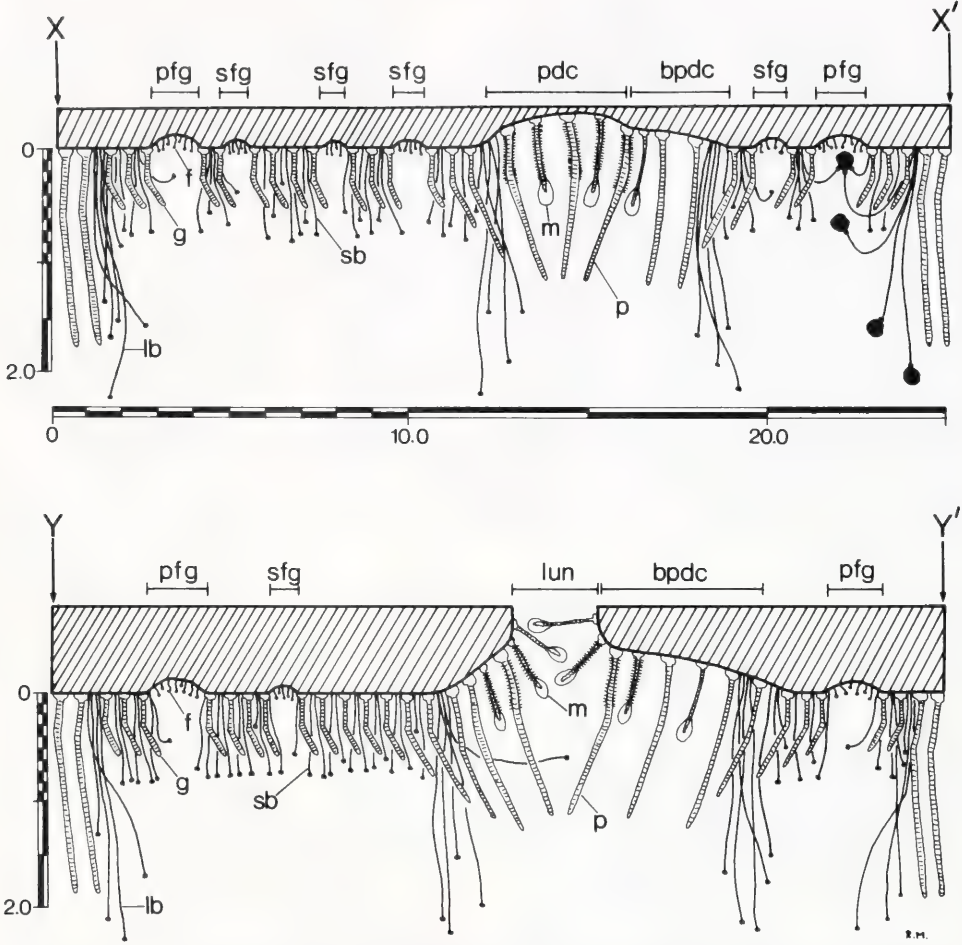

FIGURE 4. Transects through oral ambulacrum of *Mellita quinquiesperforata* shown in Figure 3A. Scale bars in mm. Vertical exaggeration X3 (except for lunule miliary spines). At right side of XX′ is a sequence showing long barrel-tipped podia collecting and placing substrate particle in food groove, aided by short barrel-tipped podium. bpdc, branch of pressure drainage channel; f, food groove podium; g, geniculate spine; l, locomotory spine; lb, long barrel-tipped podium; lun, lunule; m, miliary spine, p, pressure drainage channel spine; pdc, pressure drainage channel; pfg, primary food groove; sb, short barrel-tipped podium; sfg, secondary food groove.

2G), there are five pairs of buccal podia, 1.5 mm long (Fig. 2F) with bluntly rounded tips.

Currents powered by bands of cilia along the spine shafts occur between all spine types. Rates of ciliary flow are similar in the different spine fields (0.8–0.9 $\text{mm} \cdot \text{s}^{-1}$), but rates of particle movement may be different and depend on the spacing between spine shafts and podia (Table II). Velocities were estimated for particles 10 and 30 μm in diameter because these sizes are common in gut contents and are in the size range favored by the sieve hypothesis. On the aboral surface the currents are centrifugal except at the margins of the lunules, where there is a very narrow region (comprising

TABLE II

Velocities ($mm \cdot s^{-1}$) of ciliary borne particles of 10 μm (velocity 1) and 30 μm (velocity 2) and inter-obstacle spacing (mm ± S.D.) in different regions of the surface of Mellita quinquiesperforata

Spine field	Obstacles	Space	Velocity 1	Velocity 2
Locomotory	L–L (20)	0.46 ± 0.087		
	L–M (20)	0.32 ± 0.095	0.85 (9)	0.60 (9)
Geniculate	G–G (25)	0.15 ± 0.042		
	G–P (25)	0.05 ± 0.021	0.60 (6)	0.30 (5)
PDC	S–S (25)	0.40 ± 0.085		
	S–M (25)	0.18 ± 0.027	0.70 (7)	0.70 (7)
Ambitus	F–F (20)	0.13 ± 0.041		
	F–P (20)	0.04 ± 0.023	—	—
Aboral	C–C (34)	0.20 ± 0.044		
	C–M (34)	0.10 ± 0.011	0.85 (9)	0.64 (8)

Obstacles: (i) Spine types: L = locomotory; G = geniculate; M = miliary; F = fringe; C = club; S = pdc spine. (ii) P = podium. Number of estimates of particle velocities are given in parentheses; all estimates were ±20%.

about 2–3 spine rows) in which the flow is diverted towards the lunules. At the ambitus the flow is both centrifugal and downward. On the oral surface the pattern of ciliary flow is much more complex than has been supposed by previous writers. Within the locomotory spine fields and the pdc's the flow is strongly divergent towards the adjacent geniculate spine fields (Fig. 5), but the center of the fields include a small centripetal component. Flow traverses the geniculate spine fields from the pdc side towards the locomotory spines, passing across the food grooves without interruption. At the junction between geniculate and locomotory spine fields (Fig. 5), the currents run directly into each other. As a result, both currents turn down towards the substrate. In contrast, at the junction between pdc and geniculate spine fields, some flow from the pdc enters the geniculate area and joins the flow towards the locomotory spines and some is deflected downwards (Fig. 5, inset). Towards the center there remains a slight centripetal flow which continues into the peristomial region. As elements of this flow converge beneath the mouth itself, they are united in a downward flow towards the substrate. The various downwardly directed flows are turned horizontally and, depending on the details of sediment contour, flow radially beneath the pressure drainage channels towards the lunnules and the ambitus (Fig. 5, inset). With an inverted microscope it is perfectly easy to see centripetal flow within the pdc spine fields and centrifugal flow beneath them. Locomotory movements of the spines and occasional "slumping" of the body (Lane, 1977) periodically interrupt the sediment surface flows so that outward flow via the lunules or at the anterior ambitus is, at best, intermittent. Indeed, periodically flow through the anal lunule may be temporarily reversed, water passing downward as the animal raises itself above the substrate.

Small particles caught in the ciliary flow on the aboral surface are carried to the ambitus, passing between the spine shafts. At the ambitus, many are lost simply by continuation of their centrifugal motion. A small number of particles pass around the ambitus and enter the oral surface currents. The bulk of this flow crosses the borders of the geniculate spine fields at some point, as described above. These spine fields are

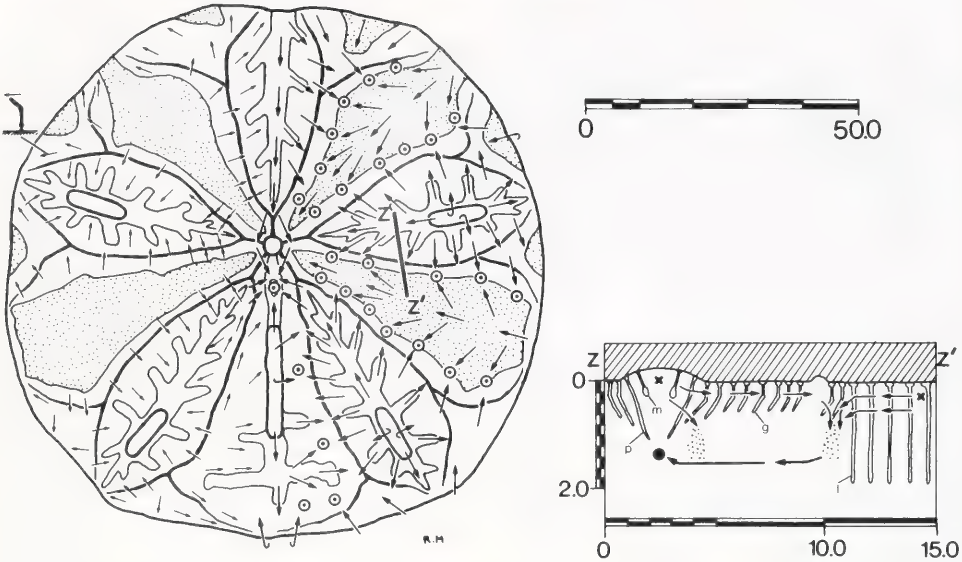

FIGURE 5. Ciliary currents on oral surface of *Mellita quinquiesperforata*. Larger arrows on right side of figure indicate direction of flow, dotted circles show meeting of currents resulting in particle dump sites. Smaller arrows on left side show direction of bend in geniculate spines (as indicated at upper left), locomotory areas stippled. Inset shows transect ZZ'. Crosses indicate flow into page, large dot indicates flow out of page (towards lunule), stipples represent deposition of particles. See text for further explanation. Spines: g, geniculate; l, locomotory; m, miliary; p, pressure drainage channel. All scale bars in mm.

bordered by long barrel-tipped podia, which occur nowhere else (Figs. 3B, 4). As the flow enters the bands of podia, it is slowed and directed downwards. The podia are richly supplied with mucus glands and suspended particles are rapidly trapped or dropped to the substrate. Those which are caught in mucus are often, but not always, incorporated in the streams of material sent to the mouth and eventually may be ingested.

Feeding was observed with natural substrate and with diatom enriched sediments. Unlike ciliary current flow, feeding is an intermittent activity. An individual may rest quietly for some hours and then feed rapidly for a few minutes, or possibly even for an hour or two. When animals are resting without feeding, no material passes along the food grooves. During these periods, fine particles caught in mucus are sloughed off. Feeding in *M. quinquiesperforata* starts when long barrel-tipped (b-t) podia pick up particles from the sediment. Long b-t podia of a sand dollar in its proper orientation collect particles, usually diatoms, diatom clumps or nutrient coated sand grains. Short b-t podia contribute little to the initial collection of particles. They function as the first stage in the transport mechanism, taking particles from the long b-t podia and passing them towards the food grooves, (Mooi, 1983; 1985a). During this process, particles are gradually coated with mucus secreted at the tips of the podia, and become increasingly adherent, so that, in the food grooves, they form well defined mucus-bound cords. Food groove podia contribute substantially to the formation of the cords and are also responsible for their eventual transport to the mouth. Within 20 seconds of the start of feeding, cords have started down the food grooves to the mouth. As the cord advances, downstream food groove podia are stimulated into vigorous swinging.

The average speed of the cords within the grooves is about 0.16 mm · s^{-1}. Large food groove podia, absent in juveniles, are most readily identified in specimens >20 mm in diameter. As the mucus cord approaches the rim of the peristome, the large food groove podia guide the strand between the circum-oral spines to the buccal podia, which in turn steer the entire cord into the mouth. No evidence of particle rejection was observed at the peristome, nor elsewhere. The feeding process could sometimes be initiated in a resting sand dollar simply by supplying diatom-rich material. Ambital accessory podia started exploring the material within a minute or so, picking up and discarding particles. The animal became more active and as the barrel-tipped podia contacted diatoms, food collection started. The accessory podia did not appear to be directly involved in selecting particles, neither are they another part of the transport system, their role appears to be primarily sensory. The overwhelming bulk of the collection was by the unaided long b-t podia. At no time during feeding did we see large diatoms carried in the ciliary currents and feeding was never initiated by fine particles supplied to the aboral surface.

The natural substrate of Bird Shoal is a very well sorted, medium to fine quartz sand with over 90% of the particles by number measuring 100–400 μm (mean 180.78, S.D. ± 59.74). Only 8.7% of the particles were less than 100 μm. No statistically significant differences were found between replicates and the data were pooled (Fig. 6). Grains were angular to sub-angular, with a mean elongation (width/length) of 0.69. Very little shell debris was observed. Acid soluble carbonate was estimated to be <1% by weight. Several species of unattached diatom (Table III) were identified but they make up only a small fraction of the total number of particles (≪1%). However, SEM and light microscopy showed that most of the sand grains support growths of smaller diatoms and were partially coated with organic material, as observed in other studies (*e.g.*, Telford *et al.*, 1983). During feeding, sand dollars select diatoms in greater proportion than their occurrence in the sediment. They rapidly picked out large diatoms when these were offered, and, although the ratio of diatoms to inorganic particles in our plankton enriched substrates was 1:4, in the food grooves between 90% and 100% of the particles were diatoms. Examination of the food groove material confirmed the high incidence of diatoms when sand dollars were feeding in native sediments. Otherwise, we were unable to detect any preference in the food material. Inorganic particles were collected in the proportions in which they occurred in the sediment (Fig. 6). Within the digestive system, very different proportions were found (Fig. 6): over 97% of the particles by number were less than 100 μm. Analysis of variance shows that these differences are statistically significant ($P \ll 0.001$): the mean particle size in the gut is smaller and the distribution of sizes is different. In addition to broken diatom frustules, much of the gut material was sharp, angular sand granules, similar to those

TABLE III

Diatom forms, sizes, and relative abundance in sediment samples from Bird Shoal, North Carolina

Form	Genus	Size, μm	Abundance
Centric, single	*Actinocyclus*	100–175	Sparse
Triangular, single	*Biddulphia*	75–125	Sparse
Centric, single	*Coscinodiscus*	75–150	Abundant
Naviculoid, single	*Rhaphoneis*	to 150 × 100	Common
Naviculoid, colonial	*Striatella*	to 80 × 40	Common
Centric, colonial	*Thalassiosira*?	50	Common

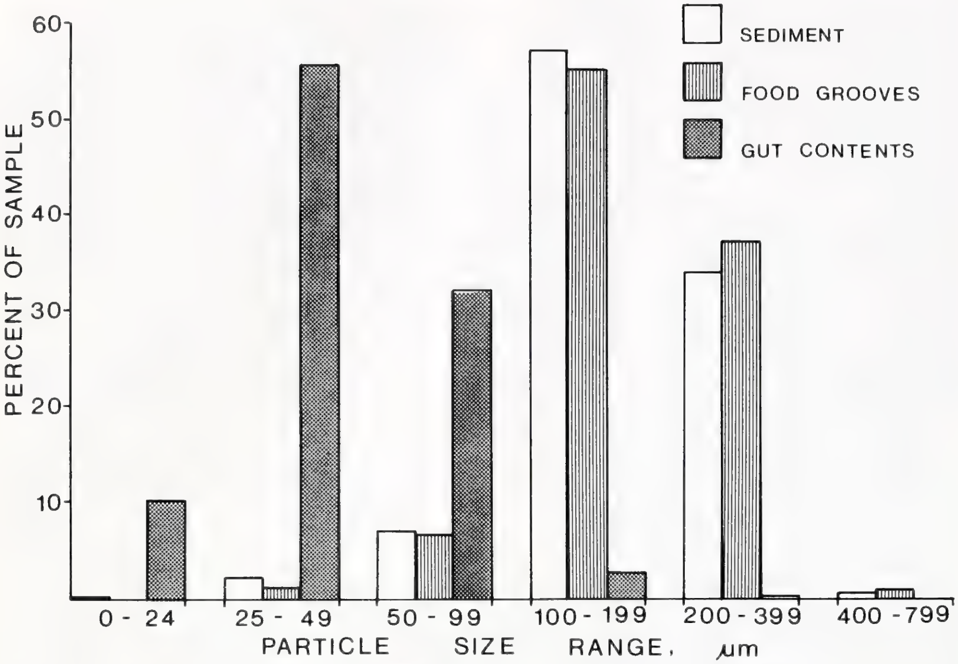

FIGURE 6. Percent composition of different particle sizes in the natural substrate, food grooves, and gut contents of *Mellita quinquiesperforata*.

from freshly fractured particles. Among the larger particles, we were able to identify many of the more common species of diatoms, including those shown in Table III.

DISCUSSION

Since the work of McGinitie and McGinitie (1949) surface ciliary currents have featured prominently in explanations of feeding in clypeasteroids. Precise description and mapping is difficult because the currents are often quite feeble and subtle changes in direction are not always apparent under the microscope. Goodbody (1960) made no attempt to map the oral surface currents in *L. sexiesperforata,* stating only that they carried suspended particles to the food grooves. Only in the center of each locomotory spine field and each pressure drainage channel, is the flow centripetal. On either side of this, the flow is strongly divergent towards the geniculate spine fields. This departure from centripetal flow in *M. quinquiesperforata* has not been mentioned by previous investigators (such as Ghiold, 1979; Alexander and Ghiold, 1980; Smith and Ghiold, 1982). These writers described the flow as strictly centripetal. However, centripetal flow cannot terminate at the center, it must continue somewhere. The principle of continuity (see Vogel, 1981, *inter alia*) can most simply be understood as the common sense notion that if a volume (X) of water enters a pipe or system of pipes, then the same volume (X) must leave the system. In a continuous flow, the principle is expressed using flux which, for a single pipe, is the product of cross-sectional area at the entrance and the average velocity of the entering fluid. The flux of fluid entering a system of many pipes is likewise equal to that leaving:

$$\sum_{i}^{n} S1_i \cdot U1_i = \sum_{j}^{m} S2_j \cdot U2_j$$

where $S1_i$ and $S2_j$ are the input and output areas, respectively, and $U1_i$ and $U2_j$ are their respective velocities. The variables of summation, $i = 1$ to n, $j = 1$ to m, indicate the numbers of input and output pipes or channels. The complex series of spaces between the spines of sand dollars may be likened to the above system of pipes and this allows us to draw some important conclusions about ciliary flow. On the aboral surface, ciliary flow is centrifugal, at $0.8\ \text{mm} \cdot \text{s}^{-1}$. With increasing distance from the center, the perimeter, and hence the cross-sectional area of the flow, increases. Since the rate of flow is undiminished, the principle of continuity requires that more fluid enter the system. This is accomplished via the very small spaces between the miliary spine sacs and expanded tips of the club spines. Approaching the ambitus there is a narrow band of accessory podia between the spines, which effectively decreases the sectional area of flow. Accordingly, flow must either be accelerated or diverted. In practice, both responses can be observed: flow is diverted away from the test surface, *i.e.*, a centrifugal component slightly upwards and outwards; at the same time, the downward flow between the spines is slightly accelerated. The extra half circlet of cilia on the bases of fringe spines, first observed in *E. parma* (Ellers and Telford, 1984), contributes to both increase in velocity and change in direction. The oral surface presents precisely the opposite situation: the perimeter, and hence cross-sectional area of flow, diminishes with proximity to the mouth, or center. Since the velocity of flow does not increase, it is clear that some flow must be diverted, it cannot be simply centripetal. Wherever streams converge, there must be a change in direction. Smith and Ghiold (1982) seemed to be aware of this when they claimed that water enters the mouth, swirls through the gut, exits via the anus and flows out through the anal lunule. We found no evidence to support this idea.

Our observations indicate that convergent currents occur in two situations: (i) a small residual centripetal current reaches the mouth region from the locomotory spine fields, and (ii) the principal flows out of the locomotory spine fields converge with currents moving across the geniculate spine areas (Fig. 5). The principle of continuity is satisfied because the system is three-dimensional: convergent currents are mutually deflected and united, downwards, towards the substrate. At the substrate surface the downward flow is again deflected, horizontally, and, depending on how the local sediment bed is shaped, flow becomes centrifugal towards the lunules and ambitus. The underside of *M. quinquiesperforata* is slightly concave and, in the natural habitat, this concavity is only partly filled with sand. The volume of this space is approximately 5–10 times the volume contained within the spine fields. Thus, when ciliary currents are directed downwards to the substrate surface and are no longer powered by cilia, they become much slower. Observation of the oral surface clearly shows that flow is brisk within the spine fields. Below the spines, flow is more sluggish, irregular, and sometimes in exactly the opposite direction. A similar situation prevails at the lunules, especially the anal lunule. Around the aboral rim, there is a downward flow within the spine field, and a gentle, occasionally intermittent or reversing, bulk flow upward, in the center of the lunule. The downward flow, within the lunular spine field, can only be drawn from close proximity to the rim (at most, 2–3 spine rows), without violating the principle of continuity.

Given the observed departures from centripetal flow, the bulk of the ciliary currents is directed towards the edges of the geniculate spine fields. Within these fields, flow is always away from the pressure drainage channels and towards the locomotory spines (Fig. 5). The geniculate spine fields are surrounded by a dangling curtain of long b-t podia. Flow from the pressure drainage channels is slowed among these podia, and deflected downwards, but it is not entirely stopped. Some flow continues into the geniculate field, actually crosses the food grooves, and unites with the current towards the locomotory spines. At the junction of geniculate and locomotory spine fields, the two flows collide. As described above, the flow is slowed, directed downwards and may become dispersed on the sediment surface. Suspended particles are brought by these flows to the long b-t podia and are dumped by the abrupt changes of direction and velocity. The long b-t podia are the principal food gathering structures. They are generously supplied with mucus and fine particles may adhere to them. Others, which fall to the substrate, may or may not be picked up later. Sometimes these fine particles became stuck to sand grains which were handled by podia, some of which were themselves dropped again. It is quite clear from our observations that some of this material, caught in mucus, can be incorporated into the food groove cords when the animals are actively feeding. However, it is obvious that much of it is lost during feeding. When the animals cease feeding, all ciliary-borne material is lost to the substrate because the podia are inactive and fully retracted within the spine fields.

Consideration of sinking rates and transit times also raises some doubt about the suitability of ciliary currents for feeding in sand dollars. The terminal velocity (U) of a spherical sinking particle far from walls can be derived from Stokes formula (see Vogel, 1981):

$$U = [2d^2g(\rho_p - \rho_f)]/(9\mu)$$

where d = particle diameter; g = acceleration due to gravity; ρ_p = density of the particle; ρ_f and μ = density and viscosity of the fluid medium, respectively. Assuming no wall effect, we estimate that a 10 μm phytoplankter (density 1.067×10^3 $\mathrm{Kg \cdot m^{-3}}$; Vogel, 1981) would sink at 11 $\mu\mathrm{m \cdot s^{-1}}$, and that one ten times the diameter would sink at 100 times that rate (1100 $\mu\mathrm{m \cdot s^{-1}}$). If the ciliary stream is 500 μm deep and flows at 0.8 mm $\mathrm{s^{-1}}$, then the 10 μm particle would take an average time of 25 seconds to drop from the flow. During this time it would travel about 20 mm horizontally, enough to bring it into the b-t podial zones but not to the mouth of a moderately large sand dollar. The 100 μm particle could travel an average of a mere 0.2 mm horizontally. Most ingested particles are sand, not plant cells, and their sinking rates are some 30–40 times greater. While this sort of estimate must be regarded with some reservation (sinking rates might be lower than this due to the proximity of spines), it appears unlikely that inorganic particles or large diatoms could be transported across the oral surface with the efficiency necessary for feeding. Therefore the only particles which might be transported by ciliary currents are small phytoplankters and we observed no evidence of their accumulation in the food grooves.

Many writers (Hyman, 1958; Bell and Frey, 1969; *inter alia*) have observed that *M. quinquiesperforata* spends periods in relative inactivity, without feeding. Our observations confirm that they feed intermittently, often with intervening hours of quiescence. When feeding starts there is a dramatic increase in spine and podial activity, the food grooves rapidly fill with mucus cords and material arrives at the mouth in two to three minutes. Considering the normally undemonstrative behavior of sand dollars, it is no exaggeration to describe this activity as a feeding frenzy! Ambital accessory podia extend to 5 mm or more around the margin, touching and exploring particles, picking them up, dropping them, or sometimes bringing them close to the body. These podia do not participate directly in food collection, they appear to be largely sensory (Mooi, 1983; 1985a). There is no systematic transfer of particls to place them on the aboral surface, nor within reach of the barrel-tipped podia. Food particles are picked up almost exclusively by the long b-t podia and passed to the short b-t podia for transport to the food grooves.

The podial mechanism described here is totally unlike any previously proposed use of podia. In a recent review of clypeasteroid feeding mechanisms, De Ridder and Lawrence (1982) stated that: ". . . the accessory tube feet can stretch above [sic] the spines, probe the sediment, or directly pick up food particles (Goodbody 1960, Culver 1961, Bell and Frey 1969, Ghiold 1979)." It is worth examining this statement with two objectives: (i) to determine its observational basis and (ii) to follow the fate of particles so collected. Goodbody (1960) noted the movements of podia ". . . with no particles attached . . ." but, though able to work only at low magnifications (30×), he went on to assert: ". . . I believe that the function of these podia is to probe the sand for very small particles of food, *i.e.*, particles of about 1 μm diameter." Although clearly unable to observe this, he then said that such particles were released into centripetal currents and carried to the mouth. Goodbody has been cited as the authority for this ever since, and his exact words have been used in some instances. We are unable to comment on the contribution of Culver, cited above, because we have not seen his work (M. A. thesis, Duke University, 1961). Bell and Frey (1969) made some fortuitous observations on particle movements across the oral surfaces of four inverted sand dollars. Careful reading of their account shows that they saw podial involvement only in the food grooves. Elsewhere on the oral surface their description, which is vague, mentions only spines. So far as food collection was concerned (in contrast to its transportation) they frankly speculated: "The tube feet may also be important in the initial phase of food gathering, although this activity was not observed." In a somewhat superficial treatment of burrowing and feeding, Ghiold (1979) gave no hint of his methods. Ghiold says that his account is based on personal observations (unexplained) and those from the literature (citing Goodbody, Bell, and Frey). When he asserts that: ". . . the podia concentrated on the oral surface are continually probing the sediment for food," one can only assume that this was derived from Goodbody's similar description. Subsequently, Ghiold (Smith and Ghiold, 1982) has argued that: ". . . mellitids feed almost entirely on aborally derived material." This opinion has been shared by Lane (1977) and, more recently, by Lane and Lawrence (1982): "*Mellita quinquiesperforata* ingested the very fine particles that either fell on the aboral surface of the animal or were sorted from the sediment which covered its back. The oral and marginal surface and appendages (tube feet and spines) had no part in gathering food." Thus, in previous accounts we can find no clearly demonstrated role for the podia during feeding, except within the food grooves. Starting with Goodbody (1960), perpetuated by Ghiold (1979) and most recently by De Ridder and Lawrence (1982), there have been hints and suspicions about the activity of podia during feeding, but always inextricably linked with the sieve hypothesis. However, there has been a general tendency to discount podial activity in recent years, especially in the work of Ghiold and of Smith and Ghiold (cited above).

Diatoms, which are rare compared to mineral grains, are actively selected so that they become concentrated four or five fold in the food grooves. Most of the particles collected are sand grains which are taken in exact proportion to their occurrence in the sediment. The native sediment and food groove material have identical particle size distributions, and these differ greatly from the gut contents. Within the gut, a preponderance of small particles was reported by Goodbody (1960), Moss and Lawrence (1972), Lane (1977), and by Lane and Lawrence (1982), and has generally been interpreted as evidence of small particle selection by the sieve mechanism. In substrate samples collected at Bird Shoal, particles <100 μm made up only 9% of the total by number (Fig. 6), corresponding to less than 1% by weight. Lane (1977) reported higher proportions of small particles in her substrate samples, with approximately 10% by weight smaller than 125 μm. Within the gut of our sand dollars, 97% of the particles by number were <100 μm (approximately 65% by weight). Lane (1977) found a similar difference between gut contents and natural sediments, where, estimating from her data, about 62% of the gut contents by weight were <62 μm. Kier (1974) observed that the gut of clypeasteroids usually contains "crushed" material. It is our contention that the lantern teeth actively break diatoms and sand grains into very fine particles. The lantern of clypeasteroids is large, heavily muscled, and has greatly hardened teeth (Kier, 1974). Most previous studies have neglected the role of the lantern in feeding. Lane (1977), however, asserted that the lantern was not involved and that: "No grinding or chewing functions were noted." (p 134). In contrast, in *Echinocyamus pusillus,* Telford *et al.* (1983) made direct observations of crushed material in the gut and scraping activity of the lantern, while Mortensen (1948), Kier (1974), and Mooi and Telford (1982) all observed crushed materials in the gut of other clypeasteroids. Furthermore, Timko (1976) specifically remarked that the teeth of *D. excentricus* ". . . thoroughly ground the food prior to swallowing." (p 252). Sediment particles broken between microscope slides yield fragments similar in size and shape to those seen in the gut. Sand grains are inherently weakened by minute fracture planes so that even the energies involved in inter-grain collisions during wind or water transport can be sufficient to break them into smaller sizes (Leeder, 1982). We suggest that the force delivered by the tips of the lantern teeth is at least equal to this. According to Leeder (1982) particle fracture in transit is negligible in grains below 50 μm because collision energies are too low. The virtual absence of particles below this size at Bird Shoal is due to continual stirring by wave action and winnowing by local currents. The frequent occurrence of crushed diatoms in the gut of clypeasteroids suggests that exposure of their contents to digestive secretions may be the principal purpose of lantern activity, and that sand grains might only be fractured incidentally.

Surface ciliary currents are ventilatory and cleansing in function. *Mellita quinquiesperforata* occurs in clean, medium grained sands and is intolerant of finer particles (Weihe and Gray, 1969). Undoubtedly, some material swept from the surface in ciliary currents is incorporated in the food streams. However, experiments with carmine particles yield very misleading results. Sand dollars live in sediments with low organic contents. When carmine particles are used to track ciliary currents, they are applied in amounts far exceeding the natural occurrence of organic matter of that particle size. Observations of the underside of sand dollars, even when buried, show that the surface is perfectly free of fine particulate material: there is none adhering to spines and podia. When carmine is added, the podia rapidly become entangled in mucus-bound particles. This is so different from the natural condition that it must be regarded with the utmost caution. In our opinion, it does not represent the manner in which small particulate matter is dealt with in feeding, nor do ciliary currents make a significant contribution to this process.

In summary, our observations show conclusively that food material is collected by oral surface podia. We cannot categorically rule out the inclusion of ciliary borne particles, but we are of the opinion that ciliary currents ventilate and keep the surface free of particles and, in so doing, make at most a trivial contribution to feeding. We are persuaded that the proponents of the sieve hypothesis have mistaken the cleansing activity for feeding, and have never witnessed the real process of food collection at all. We now challenge all "sievists" to provide convincing data in support of their hypothesis. Despite suggestions to the contrary (Ghiold, 1979; Lane and Lawrence, 1982; and others), we have found no evidence that the spines of *M. quinquiesperforata* secrete mucus. Our direct observations support the functional explanation of the histology, diversity, and distribution of podial types, described in detail by Mooi (1985a, b). The podial mechanism of feeding proposed here is strikingly like that of other clypeasteroids recently examined, for example *Echinarachnius parma* (Ellers and Telford, 1984), *Echinocyamus pusillus* (Telford *et al.*, 1983) and, to some extent, *Dendraster excentricus* (Timko, 1976). Finally, this account of feeding provides a role for the large and well developed lantern which, under the sieve hypothesis, has been assigned no function in feeding.

ACKNOWLEDGMENTS

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THE FINE STRUCTURE OF THE AMEBOCYTE IN THE BLOOD OF *LIMULUS POLYPHEMUS*. I. MORPHOLOGY OF THE NORMAL CELL

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ABSTRACT

The fine structure of the amebocyte (hemocyte) in the blood of *Limulus polyphemus* was reinvestigated regarding its normal state to resolve existing discrepancies in reporting before proceeding to studies of its participation in blood coagulation. The ovoid cell has all the organelles of a well equipped, metabolically active cell; having a nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomes. However, no mitotic figures were seen. The most distinguishing characteristic of the cell is the presence of a population of large granules (predominant) and small, asymmetric dense granules (not always present). The large granules originate from the Golgi apparatus and the matrix of the developing granules undergoes a wide range of transitional patterns before assuming the mature dense condition. The transitional stages are interpreted as formative of new granules rather than stages of degranulation as previously reported. The small, asymmetric dense granules are considered to be a type of granule distinct from the larger more predominant type of granule.

INTRODUCTION

The blood coagulation mechanism of *Limulus polyphemus,* the horseshoe crab, is notable because of its marked sensitivity to the endotoxins produced by gram-negative bacteria. The initial observations leading to this important discovery were reported by Frederik B. Bang (1956). Within two decades the original investigations have been extrapolated into many areas of basic and clinical research (Cohen, 1979). Clot formation is dependent on a characteristic hemocyte that discharges its granules as part of the phenomenon (Levin and Bang, 1964a, b; Levin, 1967; Murer *et al.,* 1975; Ornberg and Reese, 1981). In preparation for ongoing studies of factors that may either stimulate or inhibit clot formation, the fine structure of the normal hemocyte was examined in order to obtain control or base line observations. The most complete description to date of the fine structure of the native, unstimulated hemocyte in *Limulus* has been provided by Dumont *et al.* (1966). Our observations and, particularly, our interpretations differ sufficiently from theirs and warrant the new study of the normal cell reported here. Since the *Limulus* hemocyte is motile (Armstrong, 1980), it is customarily referred to as an "amebocyte." That nomenclature is retained here. For a comparative review of the blood cell types in a range of invertebrates, including insects, see Ratcliffe and Rowley (1979).

MATERIALS AND METHODS

Horseshoe crabs (*Limulus polyphemus*) were obtained from the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, and

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kept, with feeding, in running seawater a week or more before being used. Fixing of the normal amebocytes in the blood was performed as described below.

A short 14 gauge hypodermic needle was thrust through the cephalothorax-opisthosoma flexure into the cardiac space. The blood expressed by cardiac pressure was allowed to flow directly from the needle to form a thin layer in the bottom of a petri-dish filled with fixative. The flexure was first cleansed with alcohol. The needle and the dishes were cleansed and heat sterilized at 180°C for 4 hours to eliminate possible endotoxins. The resulting clot was loosened and the upper surface layer, which was fully exposed to fixation, was dissected free to a depth of about 1.5 mm and trimmed prior to postosmication.

The primary fixative that proved best was 2.5% glutaraldehyde in 0.065 *M* Millonig phosphate buffer to which 0.7% tannic acid was added and the pH adjusted to 7.4. The tannic acid was the low molecular weight product manufactured from a Turkish Aleppo nut gall in Belgium (Simionescu and Simionescu, 1976) and sold by Mallinckrodt., St. Louis, Missouri (stock number 1764 and specify lot EAH).

Fixation was initiated at room temperature for about one half hour and completed for an additional three to four hours in a refrigerator (4°C). The tissues were then rinsed several times in cold 0.065 *M* phosphate buffer and placed in a cold mixture (4°C) of 2% aqueous osmium tetroxide (1 part), 2% potassium ferrocyanide (1 part), and 0.13 *M* phosphate buffer (1 part). The osmication vials were transferred to a slanted, rotating table and, for 45 min, allowed to come to room temperature. A brief rinse in 0.065 *M* phosphate buffer was followed by repeated rinses in distilled water, then dehydration and embedment in Epon 812. Thin sections were stained with uranyl acetate and lead citrate.

Supplemental observations of living amebocytes were made utilizing video-enhanced contrast, differential interference contrast microscopy, as described previously (Richardson *et al.,* 1983).

RESULTS

The *Limulus* amebocyte in its normal state is a prolate disc that is sometimes modified toward a fusiform or spindle shape. It is readily identified by having many large granules and a centrally located nucleus (Fig. 1). The cytoplasmic matrix itself is packed with small ribosomal-like particles. A prominent circumferential band of microtubules is present and probably serves as a physical aid to preservation of shape. The band is not illustrated here because it does not differ from the bands described in the blood cells of many other species (Meves, 1904; Fawcett, 1959; Fawcett and Witebsky, 1964; Dumont *et al.,* 1966; Nemhauser *et al.,* 1980).

The large membrane bound granules (Fig. 1) are the most prominent and characteristic feature of the amebocyte. In the context of electron microscopy, the granules might better be designated "bodies" because of their large size, but the classical histological term "granules" will be used here. Two types of membrane bound granules were observed. The major population is composed of large spherical, sometimes ovoid granules. A minor population is composed of smaller, ellipsoid granules. We refer to the former as "major granules" and the latter as "minor granules."

Most of the major granules have a dense, evenly stained matrix. However, a few exhibit varying degrees of density with the matrix ranging from unorganized clumps to swirls of rod-shaped material that are compacted and finally fused into the homogeneous matrix seen in the mature granule. These are most frequently found in the neighborhood of the Golgi complex and we interpret them as "transitional granules." *i.e.,* formative stages of the major granule (Figs. 2–5, 8).

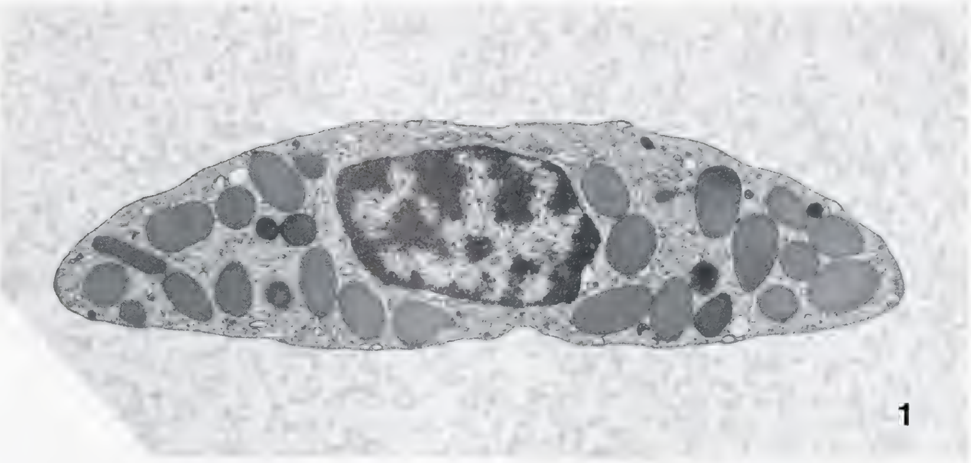


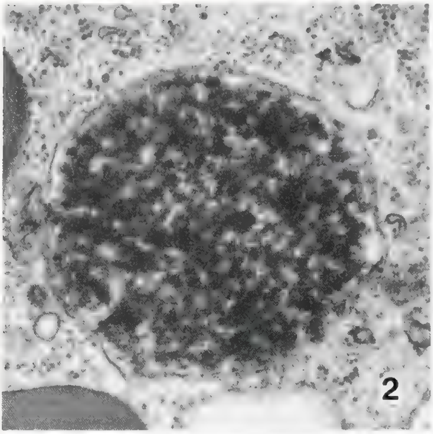
FIGURE 1. Longitudinal section of a *Limulus* blood amebocyte. In this section the cell is spindle shaped. A longitudinal section cut at right angles to this one would reveal a more oval shape. Note the characteristic large, homogeneous secretory granules. 7000×

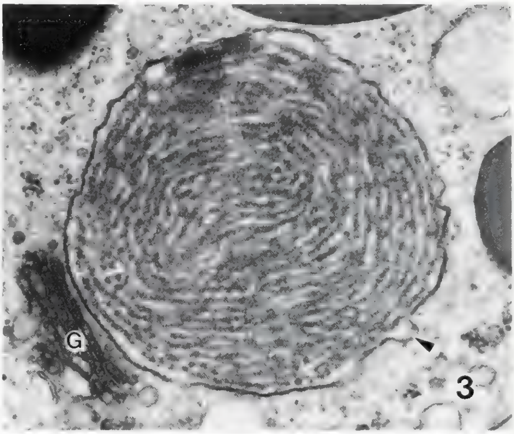
The minor granules were not observed in all of the amebocytes but were seen often enough and are so unique in their morphology that they cannot be ignored (Figs. 6, 7). The central core of the minor granule is quite dense, more so than the matrix of the major granule. Most characteristic is a relatively clear zone at each end of the ovoid-shaped granule. No recognizable transitional stages of the minor granule were detected. There is no indication of its ultimate fate during blood coagulation. Very dense vesicles are sometimes produced by the Golgi apparatus and very probably contribute to formation of the minor granule.

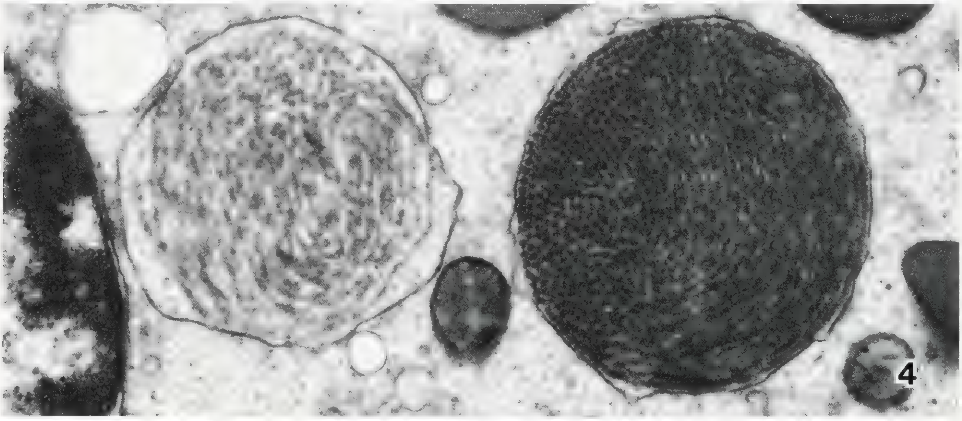
Phase and differential interference contrast microscopic studies of living amebocytes revealed that the major granules have slow random movements. However, the minor granules often appear to move on long, linear paths which may be retraced immediately.

Many of the amebocytes have a well developed Golgi apparatus (Fig. 8). Due to the limitations of the plane of sectioning, it cannot be said that all of the amebocytes have an apparatus but indeed they are quite common. We never observed more than one Golgi apparatus in a cell though the cell may become quite large. Sometimes the complex may give rise to aberrant views dependent on plane of section. For example, a well developed apparatus may be curved into funnel shape (Fig. 9). Sections paralleling the top of the "funnel" demonstrate a circular or semi-circular stack of membranes (Fig. 10). Consequently, sections parallelling the long axis may present what appears to be two separate apparatuses associated at an angle. The *cis* face of the complex is to the outside of the funnel and the *trans* face is to the inside. The *cis* face is associated in normal fashion with elements of the rather sparse rough endoplasmic reticulum (Figs. 8, 9). The *trans* face, again in classic fashion, buds off small particulate filled vesicles that fuse into larger vesicles which finally form a transitional stage of the major granule type. Occasionally a few very dense vesicles are seen (precursors of the minor granule?).

Some of the secretory vesicles of the Golgi apparatus appear to leave the immediate zone and are found scattered throughout the cell. These may assemble in other parts of the cell to form transitional granules (Fig. 5). The evidence for this is suggestive but not conclusive. The transitional stages of the major granules that are sometimes







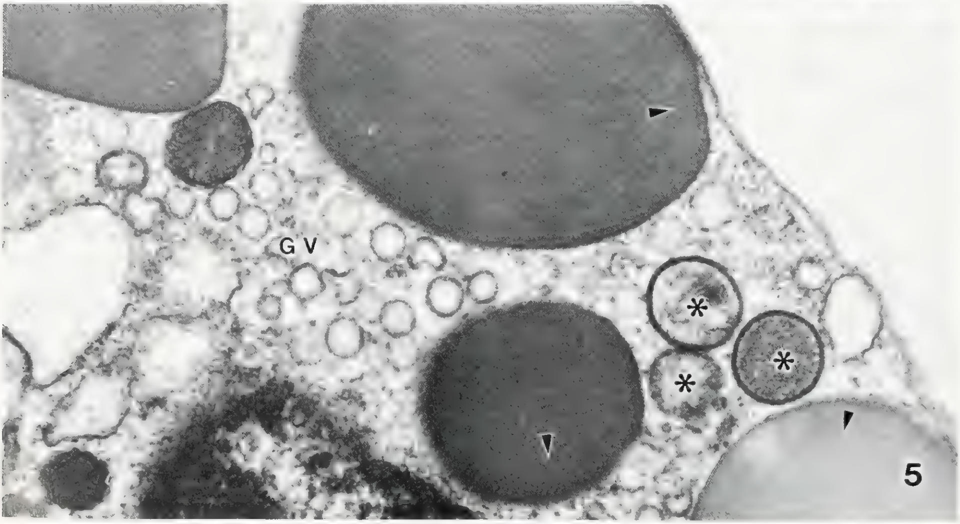
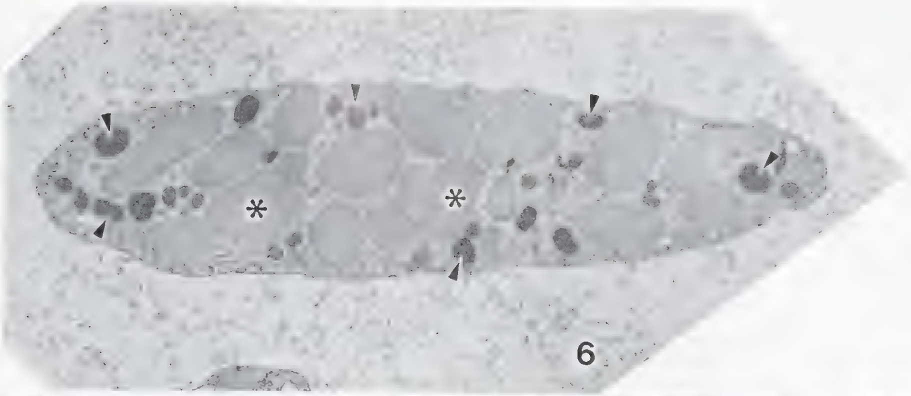


FIGURE 2. An early, amorphous stage in development of a secretory granule in the amebocyte of *Limulus*. 25,600×

FIGURE 3. An intermediate stage in the maturation of a secretory granule in the amebocyte of *Limulus*. The original flocculent material has condensed into roughly rod-shaped material. Note the attendant Golgi apparatus (G) and evidence of vesicle accretion (arrow). 22,490×



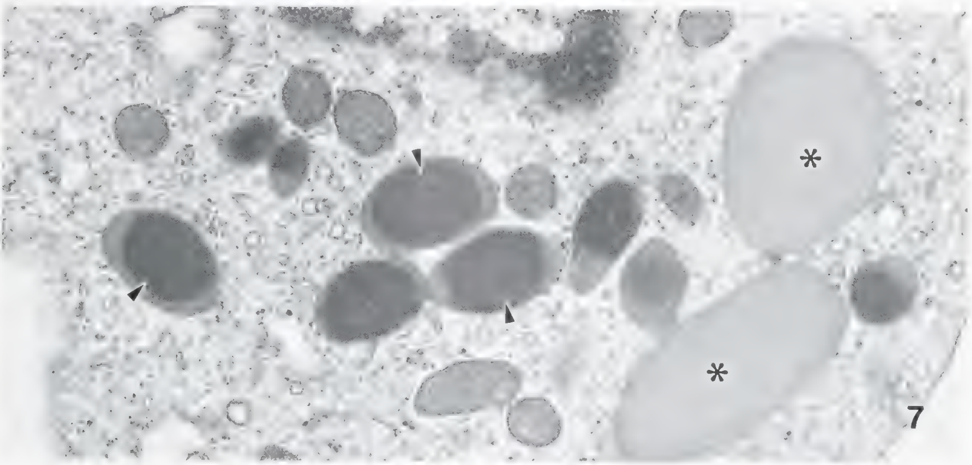


FIGURE 6. *Limulus* amebocyte showing both major (asterisks) and minor (arrows) granules. Note the marked density of the latter. 6140×

FIGURE 7. Detail of the two types of granules found in *Limulus* amebocytes. Major granules are marked with asterisks and the minor granules with arrows. 25,230×

distant from the Golgi zone might instead arrive there secondary to the random movement of the total population of granules.

Although not numerous, mitochondria are commonly found. Their dimensions vary from spherical (majority) to an elongated sausage-shape (minority). The matrix surrounding the cristae is quite dense and tends to obscure their membranes.

The amebocyte does not have a well organized endoplasmic reticulum. There is no identifiable tubular smooth endoplasmic reticulum. Irregular and sparse elements of the rough endoplasmic reticulum can be identified by the surface coating of ribosomes and the presence of more or less particulate material in the lumen. When seen,

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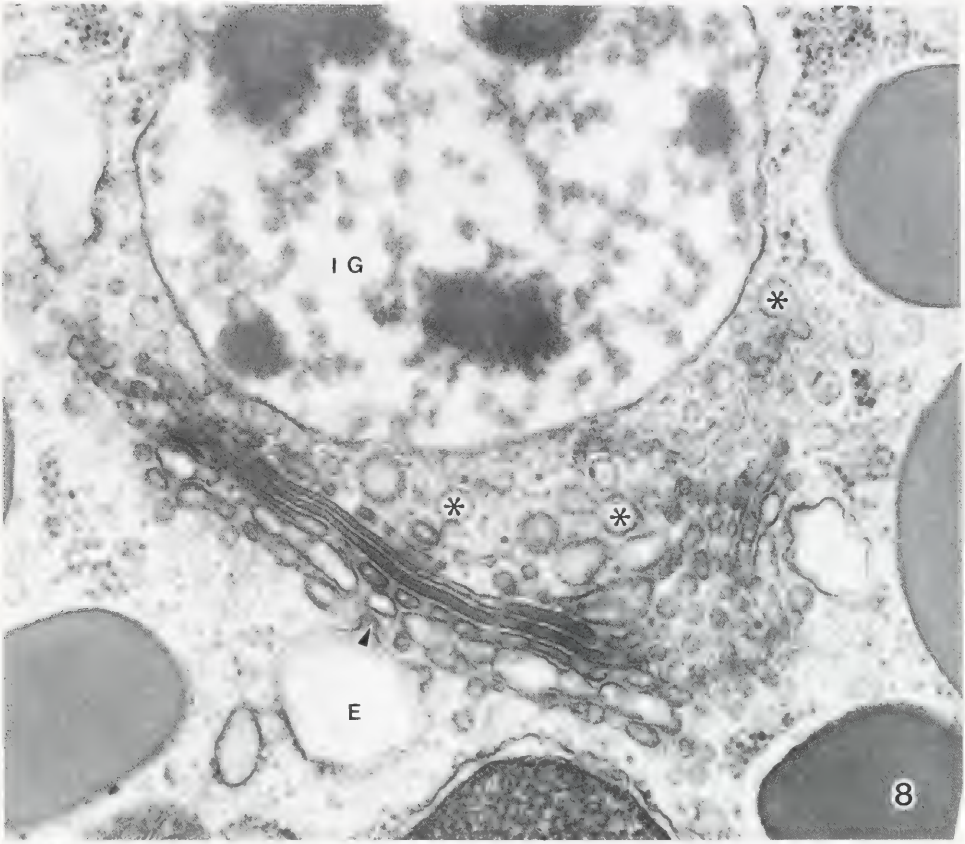
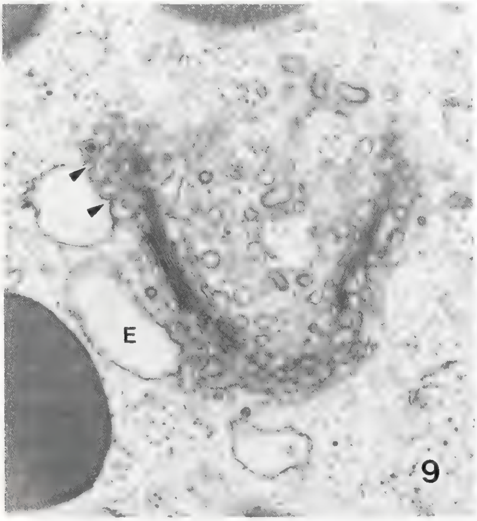
FIGURE 4. Two intermediate stages in the maturation of the secretory granules in the amebocyte of *Limulus* blood. Left is an earlier stage and right is an advanced stage, the matrix being condensed into discrete rods. 25,790×

FIGURE 5. Granules of the amebocyte in *Limulus* shown in final stages of maturation. The matrix has become compacted into dense, intimately aligned rods. The rods have a less dense core (arrows). Typical Golgi secretory vesicles (GV) are present. Also seen are vesicles (asterisks) that may serve to accrete major secretory granules distant from the Golgi apparatus (see text discussion). They are characterized by a dense layer of material immediately under the limiting membrane. 34,220×





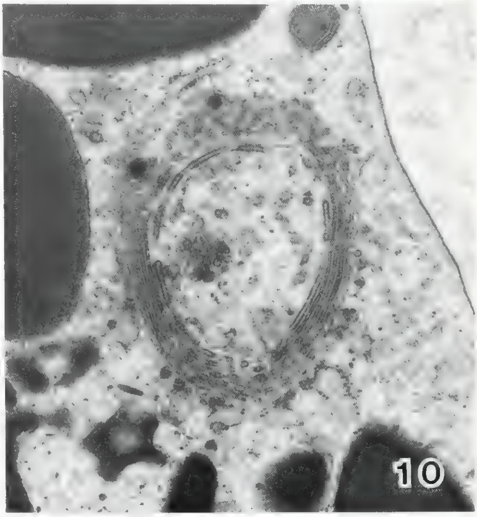


FIGURE 8. An active Golgi apparatus in the *Limulus* amebocyte. Rough endoplasmic reticulum (E) is budding material (arrow) into the *cis* face and secretory vesicles (asterisks) form on the *trans* face. A large immature intermediate granule (IG) is closely associated. 49,900×

the rough endoplasmic reticulum is usually adjacent to the cell surface, and, of course, in the Golgi zone. There is no obvious outer layer or zonation of the cell that might indicate preferential or obligatory location of rough endoplasmic reticulum. Its location near the cell surface may be merely the result of the random movement of the densely packed granules forcing it to that position.

The cytoplasmic space in the normal amebocyte is filled with densely packed particles. They frequently are joined in chains resembling the glycogen in the spinal glycogen body of chicks (Vye and Fischman, 1972). At higher magnification the particles resemble the alpha glycogen particles as classified by Drochmans (1962). The particles also morphologically resemble ribosomes (polysome type). Preliminary histochemical tests with a-amylase and RNAse controls indicate that the cytoplasmic particles are of a ribonuclear protein nature but that is still under investigation.

DISCUSSION

The *Limulus* amebocyte is an interesting blood cell because it has all the organelles of a well equipped, metabolically active cell: a nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomal particles. Nevertheless, it must be considered to be a "terminal" cell, origin unknown, because at no time were mitotic apparatuses seen in the circulating forms.

Dumont *et al.* (1966) present in good detail some of the morphology of the transitional granules described in this article. However, they concluded that the various stages represent chemical and morphological breakdown of the dense, homogeneous matrix into the dispersed, less dense vacuolar material that is finally expelled from the cell in the process of degranulation. Present evidence does not support that view and suggests that the transitional granules are formative stages of the dense major granule.

First, if the sequence of morphological stages represents a series of complex physicochemical reactions, it is unlikely that they could occur within the time frame of the almost explosive degranulation of the cell. Instead, the pleomorphic stages could represent an orderly, time consuming synthesis of material into the final major granule type. Secondly, the ubiquitous presence of all forms of the transitional granules adjacent to the Golgi zone suggests that they originate there. The occasional transitional granule in some other part of the cell could be explained by the random movement of all of the cellular granules. There is also some evidence that a few transitional granules may be assembled from small secretory vesicles that have migrated away from the Golgi zone.

As the result of experiments yet to be reported, it is our opinion that the major granule goes very abruptly from a dense phase to a finely particulate less dense phase as the cell degranulates. Normal time video-enhanced contrast, differential interference contrast cinematography by one of the authors (J.L.) has been made of endotoxin-free amebocytes. Under those specific conditions overall degranulation of the cell occurs during an extended period of time. However, the phase change undergone by any one granule is extremely rapid. In a fraction of a second a granule will expand

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FIGURE 9. A funnel shaped Golgi apparatus cut longitudinally giving the appearance of two apparatuses assembled in a "V." The rough endoplasmic reticulum (E) delivers material into the *cis* or receiving surface (arrows). 21,120×

FIGURE 10. A funnel shaped Golgi apparatus cut in cross section giving the appearance of a circular apparatus. See text for discussion. 23,050×

and lose its density. In some instances, degranulation is followed by a recoil type of movement of the cell.

That the phase change of the large granule from dense to finely particulate is always a rapid process, receives support from observations by Ornberg and Reese (1981) on ambeocytes that have been activated by a normal physiological agent, bacterial endotoxin. Using a slam-freeze cryofixation technique that immobilizes tissue in a few milliseconds (Heuser *et al.*, 1979), it was found that the phase change plus the event of exocytosis itself can occur in seconds with the phase change having no intermediate morphology(s) such as reported by Dumont *et al.* (1966).

Indirect support of our interpretation that the transitional granules are formative ones is provided by Bodammer (1978) in his study of the morphologically similar granular hemocytes of the blue crab, *Callinectes*. He interprets the various less dense granules as precursors of the mature dense granule. If it is correct that the transitional granules are indeed formative stages of the major granule and are normally present in the circulating blood, the descriptions in this report are more likely to represent the true undisturbed normal amebocyte.

The Golgi apparatus sometimes appears to be exceptionally large and active in that the layered membranes will fold around in the zone, assuming a circular or funnel shape. If the "funnel" is cross-sectioned near its mouth, the Golgi membranes appear to lie in a circle or semi-circle. If the "funnel" is sectioned longitudinally, there may be two Golgi layers of membranes tapering toward a common point. This perhaps accounts for the statement by Dumont *et al.* (1966) that several Golgi complexes may be seen in close association. We have no evidence of multiple apparatuses.

The nature and role of the oval shaped minor granules are unknown. Observations (by J.L.) of living cells reveal that an occasional small granule (minor granule?) may move in a more oriented fashion than the randomly moving larger granules. They can move in a straighter path and sometimes retrace the path. It could not be determined if such movement was associated with the circular marginal band of microtubules, there being no obvious association between the two components at the fine structural level. The clear areas of the oval-shaped granule might indicate a potential for oriented movement but we have no way of testing this possibility. Dumont *et al.* (1966) reported that small granules invade the pseudopods and retreat during active degranulation of the cell.

Many previous studies of *Limulus* have described the cytoplasmic granules (Levin and Bang 1964b; Muerer *et al.*, 1975; Ornberg and Reese 1981; Dumont *et al.*, 1966), but relatively little attention has been given to the possibility that different populations of granules are present within the cells. From the distinct differences in the morphology and the absence of detectable morphological transitions between the minor and major granules, we suggest that the *Limulus* amebocyte has two types of granules, one of which is not always present. By way of serendipidy, evidence was obtained that there may be a chemical difference between the two. The a-amylase enzyme used as a specific test for the possible glycogen nature of the cytoplasmic particles produced an unanticipated reaction in the minor granules that was not observed in the major granules. There is no currently available explanation for the particular reaction. Accordingly this subject is under further investigation.

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VOLUME REGULATION AND NITROGEN METABOLISM IN THE MURICID GASTROPOD *THAIS HAEMASTOMA*

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ABSTRACT

Ammonia and primary amine excretion and concentrations of intracellular ninhydrin-positive substance (NPS) and free amino acids (FAA) were measured in *Thais haemastoma* acclimated to salinities between 5 and 35‰ and over 14 days following direct transfer from 10 to 30‰ or from 30 to 10‰. There was no trend in excretion rates with acclimation salinity. Intracellular NPS and FAA levels were directly related to acclimation salinity, with amino acids constituting over 90% of the NPS at salinities greater than 10‰. The intracellular free amino acid pool of *T. haemastoma* was not dominated by any single amino acid but glycine, alanine, aspartate, taurine, proline, and glutamate (in decreasing order) each contributed more than 5% of the FAA. Alanine and glycine were the major intracellular osmotic effectors during both the high and low salinity transfers. Taurine levels did not change in the hyperosmotic transfer, but taurine was lost from the foot over the course of the hyposmotic transfer, suggesting that it behaves as a passive osmolyte. Snails are capable of taking up exogenous ammonia from seawater during a 10 to 30‰ transfer, suggesting that ammonia is being used as an aminating source.

INTRODUCTION

The southern oyster drill *Thais haemastoma* (Gray, 1839) is exposed in the field to both diurnal salinity fluctuations between 15 and 30‰ and extended periods of relatively constant salinity (Hewatt, 1951; Barrett, 1971). Even though its low salinity distributional limit in nature is 15‰, it will survive for over four weeks at salinities as low as 5–7.5‰ (Garton and Stickle, 1980; Hildreth and Stickle, 1980) and maintains a positive energy budget throughout the salinity range at temperatures greater than 15°C (Stickle, 1985a).

As is true of other marine molluscs, the hemolymph of *T. haemastoma* remains isosmotic to ambient seawater (Hildreth and Stickle, 1980; Stickle and Howey, 1975). The predominant labile intracellular osmolytes in marine molluscs are organic compounds. In many species studied to date, these are free amino acids (Burton, 1983), but changes in the intracellular free amino acid pool of several gastropods appear to be insufficient to account for the changes in intracellular osmolality following a change in ambient salinity (Schoffeniels and Gilles, 1972; Polites and Mangum, 1980). Inorganic ions and quaternary ammonium compounds such as glycine betaine and proline betaine have recently been identified as the primary labile intracellular osmolyte in some species (Pierce *et al.*, 1983).

The objectives of the present study were to (1) determine the degree of volume regulation and the changes in the patterns of nitrogen excretion in *T. haemastoma*

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during high and low salinity adaptation, (2) determine the changes in the free amino acid pool during adaptation to altered salinities, and (3) determine the extent to which changes in the free amino acid pool are responsible for salinity adaptation.

MATERIALS AND METHODS

Collection and acclimation of animals

Snails were collected from pilings and bulkheads in the vicinity of Caminada Pass near Grand Isle, Louisiana, transferred to Baton Rouge, and placed into 38-liter aquaria containing artificial seawater (ASW; Instant Ocean, Mentor, Ohio) of the same temperature and salinity (30°C, 27‰) as in the field. Salinity adaptation was accomplished by adding either deionized water or concentrated ASW to change salinity by 2‰ per day. Animals were held at the final salinities for two weeks before being used. Small oysters (*Crassostrea virginica*) were provided as prey.

NPS and free amino acids

Ninhydrin-positive substances (NPS) and free amino acid levels were measured in the foot tissue of snails acclimated to 5, 7.5, 10, 15, 20, 25, 30, and 35‰ at 30°C, and directly transferred from 10 to 30‰ or from 30 to 10‰. Measurements were taken on transferred snails ($n = 10$) on days 0, 1, 2, 3, 7, 10, and 14 after transfer. NPS and free amino acids were also measured in the foot tissue of snails used in the ammonia-loading experiments described later.

Foot tissue was excised, then frozen in liquid nitrogen and lyophilized. After grinding in a Wiley mill, 10 mg of tissue were leached in 5 ml of 5-sulfosalicylic acid for 48 h. Samples were centrifuged at $20{,}000 \times g$ for 15 min and the supernatant was assayed for NPS according to Rosen (1957). Concentrations of individual amino acids were determined on a Beckman Model 119 amino acid analyzer.

Ammonia and primary amine excretion and activity

The rate of ammonia and primary amine exchange was measured by incubating snails in 150 ml of ASW for 60 min and analyzing the incubation medium by the Solorzano (1969) phenol-hypochlorite method (ammonia) and North's (1975) fluorescamine technique (amines). Ammonia exchange is defined as the sum of NH_3 and NH_4^+ exchange. Since urea makes up an appreciable fraction of the excreta in some carnivorous marine invertebrates (Stickle, 1985b), further samples of the incubation medium were analyzed for urea by the Sigma urea assay (Sigma technical bulletin #640) at 10 and 30‰. All glassware used in excretion measurements had been baked at 450°C in a muffle furnace to eliminate exogenous amines. Excretion was measured for snails acclimated to each steady state salinity and at hours 3, 6, 9, and 12, and days 1, 2, 3, 4, 5, 6, 7, 10, and 14 after transfer from 10 to 30‰ or from 30 to 10‰.

Further experiments were designed to test the ability of *T. haemastoma* to take up exogenous ammonia from the medium for use as a possible aminating source during high salinity acclimation. The ammonia excretion rate of snails acclimated to 10 and 30‰ was measured using incubation water containing various concentrations of NH_4Cl up to 350 μM. These served as a control to see if snails would normally take up ammonia from ambient seawater. Then snails acclimated to 10‰ were placed in a chamber through which 30‰ water was pumped. The high salinity water flowing through the cell contained 0, 35, 45, 100, 175, or 350 μM NH_4Cl. Ammonia excretion or uptake was measured after 24 h for the 35, 45, and 100 μM spiked animals, and in the time intervals of 8–12 h and 20–24 h after the transfer for animals subjected to either 0, 175, or 350 μM NH_4Cl. Foot tissue was sampled for NPS and FAA determination from 10‰ acclimated snails (controls) and at h 12 and 24 after transfer from snails in the 0, 175, and 350 μM NH_4Cl spiked transfers.

In both sets of salinity transfer experiments, a snail's activity was assigned a value of 1.0 if its foot was extended and attached to the substrate, 0.5 if the foot was extended but not attached, and 0 if the foot remained withdrawn.

Body water determination

The amount of water in the soft tissues at each salinity was determined as the difference in the weight of the soft parts before and after lyophilyzation. Oglesby's (1975) beta value was calculated at each steady state salinity as an indicator of the degree of regulation of body water content.

Statistical analyses

The General Linear Model procedure and Duncan's Multiple Range option of the Statistical Analysis System (SAS Institute, 1982) were used in data analysis. A probability level of 0.05 was significant.

RESULTS

Steady state experiments

Levels of ninhydrin-positive substances in foot tissue of *Thais haemastoma* were directly related to the acclimation salinity over the range of 5–35‰ when expressed in terms of μmoles · g dry tissue weight^{-1} or μmoles · g tissue water^{-1} (Fig. 1).

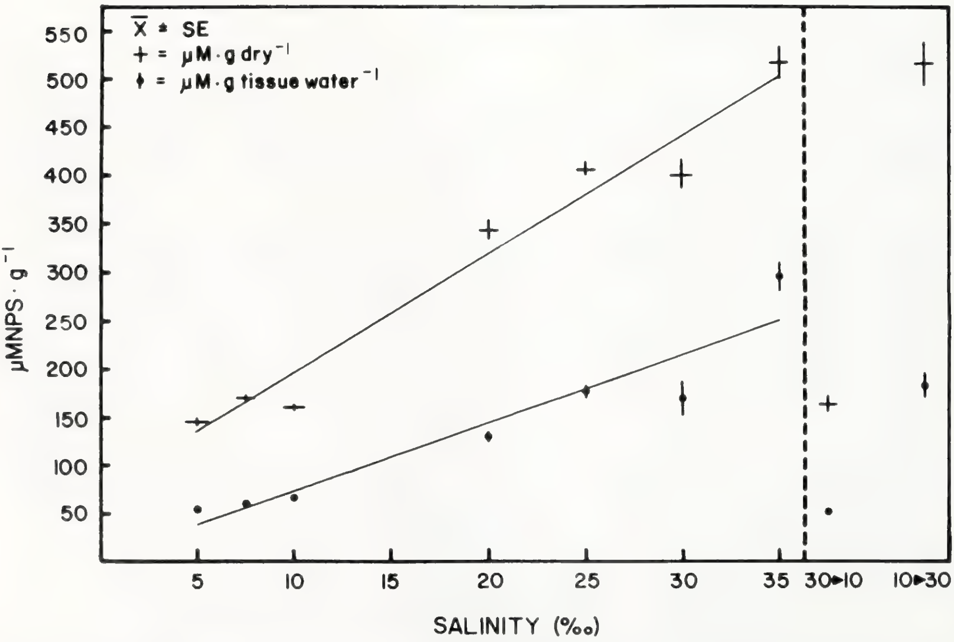

FIGURE 1. Ninhydrin-positive substance concentrations ($\bar{x} \pm$ S.E., n = 12) in the foot tissue of *Thais haemastoma* expressed as μmoles NPS · g dry tissue weight^{-1} (+) and as μmoles NPS · g body water^{-1} (●).

The free amino acid composition of the foot tissue of *T. haemastoma* at different acclimation salinities is given in Table I. Free amino acids comprise over 91% of the NPS pool at acclimation salinities of 10‰ and above. At 5 and 7.5‰ free amino acids account for 72 and 61% of the NPS, respectively. Other non-amino acid nitrogenous substances make up a significant portion of the NPS pool at very low salinities.

Excretion rates of snails acclimated to constant salinities varied among salinities (Fig. 2). Ammonia excretion rates were significantly greater than zero, but did not show a linear trend across salinities. The rate of ammonia excretion was higher at 15 and 20‰ than at any of the other acclimation salinities. The rate of primary amine exchange in snails acclimated to 5, 7.5, and 30‰ was in the uptake direction from the incubating medium. There was no significant exchange of amines between the animals and the medium at either 10 or 35‰, and the snails excreted amines at a constant rate of 0.17 μmole $\cdot$ $g^{-1} \cdot h^{-1}$ between 15 and 25‰. The rates of urea excretion at 10 and 30‰ were 0.17 ± 0.07 and 0.20 ± 0.06 μmoles $\cdot$ g dry weight$^{-1} \cdot h^{-1}$, respectively, representing a minimal contribution to total nitrogen excretion.

Thais haemastoma is an excellent regulator of tissue water content. When acclimated to constant salinity between 5 and 30‰ the percentage of the fresh weight of the soft tissues consisting of water is not significantly different, and is only slightly lower at 35‰ (Table II).

Direct transfer experiments

Changes in percent body water over the 14 days of a 10 to 30‰ and a 30 to 10‰ transfer are shown in Figure 3. During the 10 to 30‰ transfer, body water declined from 79.3 ± 0.5% on day 0 to 72.7 ± 0.9% on day 2. By day 3 the percent body water had stabilized, and was not significantly different from the 30‰ control. For the 30 to 10‰ transfer, percent body water increased from 71.1 ± 3.8% on day 0 to 80.2 ± 1.3% on day 2, was not significantly different from the 10‰ control by day 3. The beta value was 0.13 two weeks following transfer to either high or low salinity. Unlike the animals used for steady-state determinations of body water content, the snails used in these experiments showed a significant ($\alpha = 0.05$) difference in percent body water on day 0 (before being transferred) between 10 and 30‰ yielding a beta value of 0.18. This is still a very low value for beta and is indicative of excellent regulation of body water content.

Fourteen days after being directly transferred from 30 to 10‰, the concentration of foot NPS was not significantly different from the 10‰ steady state value of 161 ± 3 μmoles $\cdot$ g dry weight^{-1} (Fig. 1). Two weeks after transfer from 10 to 30‰ the concentration of NPS in foot tissue was significantly higher than the steady state value at 30‰, but not from the steady state value at 35‰ (Fig. 1).

With the exception of glutamate and taurine, the concentrations of each amino acid in foot tissue of snails directly transferred from 10 to 30‰ was higher 14 d after the transfer than in foot tissue of snails acclimated to the 30‰ steady state (Table III). The largest discrepancy between the concentrations of any amino acid between the steady state and post-transfer conditions occurred with alanine, whose concentration reached 237 μmoles $\cdot$ g dry weight^{-1} on day 3 after the transfer, as compared to only 55 μmoles $\cdot$ g dry weight^{-1} in animals acclimated to 30‰.

Arginine and aspartate were the only two amino acids whose concentration did not decline over the course of the 30 to 10‰ transfer (Table IV). For each of the other amino acids, the concentration two weeks after the hyposmotic transfer was similar to the concentration in animals maintained at 10‰, except for serine and threonine, which were not detected in the 10‰ acclimated animals, but were present in small amounts in the 30 to 10‰ transfers. Alanine, glycine, and glutamate all showed tran-

TABLE I

Concentrations of amino acids in foot tissue of Thais haemastoma *adapted to constant salinities* [*μmoles · dry weight*$^{-1}$, $\bar{x} \pm$ *(S.E.), n = 6, to nearest μmole*]

Amino acid	Salinity (‰)															
	5	%	7.5	%	10	%	15	%	20	%	25	%	30	%	35	%
P-Serine	9 ± 1	9	6 ± 1	7	6 ± 0	4	7 ± 1	2	9 ± 0	3	5 ± 0	1	6 ± 0	2	N.D.	—
Taurine	N.D.	—	3 ± 0	4	12 ± 0	8	26 ± 2	6	43 ± 2	14	42 ± 1	11	42 ± 2	11	24 ± 0	5
Aspartate	25 ± 1	24	16 ± 2	15	34 ± 3	22	48 ± 5	10	44 ± 2	14	47 ± 1	12	50 ± 2	14	32 ± 4	7
Threonine	N.D.	—	N.D.	—	N.D.	—	4 ± 0	1	5 ± 1	2	6 ± 1	2	5 ± 1	1	8 ± 2	2
Serine	N.D.	—	2 ± 0	2	3 ± 0	2	17 ± 3	4	8 ± 1	3	22 ± 2	6	15 ± 1	4	30 ± 8	7
Glutamate	3 ± 0	3	10 ± 1	10	14 ± 1	9	25 ± 1	5	26 ± 2	8	26 ± 2	7	30 ± 1	8	34 ± 7	8
Proline	N.D.	—	N.D.	—	N.D.	—	68 ± 6	15	6 ± 0	2	31 ± 3	8	32 ± 6	9	41 ± 5	9
Glycine	2 ± 0	2	2 ± 0	2	3 ± 0	2	92 ± 2	20	35 ± 4	11	92 ± 6	24	78 ± 7	21	62 ± 4	14
Alanine	6 ± 0	6	10 ± 1	9	9 ± 1	6	104 ± 1	22	31 ± 3	10	52 ± 3	13	55 ± 5	15	113 ± 9	26
Lysine	5 ± 1	5	5 ± 0	5	5 ± 1	4	4 ± 0	1	7 ± 1	2	6 ± 1	1	8 ± 1	2	6 ± 0	2
Arginine	33 ± 2	32	31 ± 2	25	43 ± 3	28	28 ± 1	6	46 ± 3	14	30 ± 2	8	29 ± 1	8	22 ± 2	5
Others	20	19	29	11	21	15	40	8	54	17	24	7	18	5	70	15
Total	105		105		153		463		314		385		369		441	

N.D. = Not detected.

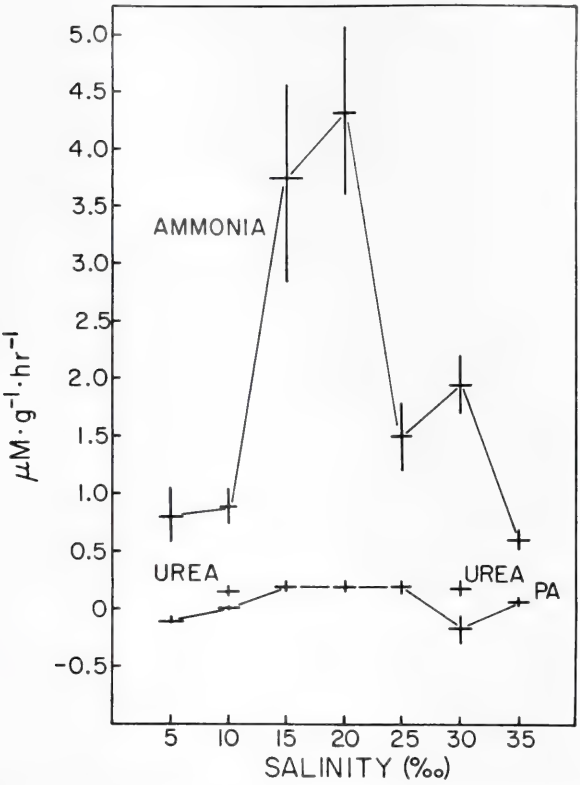

FIGURE 2. Nitrogen excretion rates (ammonia, primary amines and urea) in μmoles · g dry weight^{-1} · h^{-1} ($\bar{x}$ ± S.E., n = 12) of *Thais haemastoma* acclimated to steady state salinities.

sient increases in concentration (μmoles · g dry weight^{-1}) over the first one to two days of the 30 to 10‰ transfer. This time corresponds to the time when the snails were withdrawn into their shells with the opercula closed (Fig. 4A).

Motor activity, and primary amine and ammonia excretion all dropped to nearly zero during the 12 hours immediately following a salinity transfer from 10 to 30‰

TABLE II

Percent tissue water in Thais haemastoma *as a function of acclimation salinity.* [$\bar{x}$ ± *S.E. (n)*]

Sal	% Body water	DMR
5	72.9 ± 1.88 (12)	A
7.5	74.2 ± 0.74 (12)	A
10	70.98 ± 0.47 (12)	A
15	67.43 ± 0.57 (12)	A
20	72.34 ± 0.50 (30)	A
25	69.77 ± 0.85 (12)	A
30	72.01 ± 0.51 (24)	A
35	63.51 ± 0.84 (11)	B

Percent tissue water is not significantly different for those salinities sharing a common letter according to Duncan's Multiple Range Test (DMR).

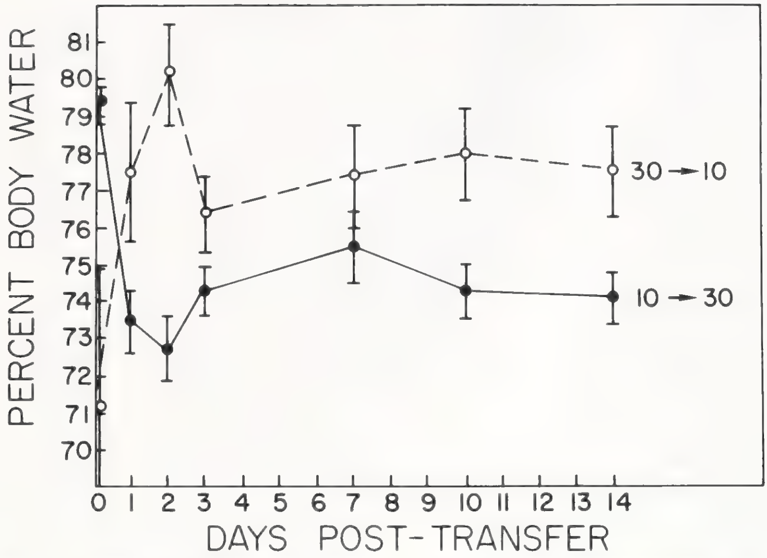

FIGURE 3. Changes in the percent body water ($\bar{x}$ ± S.E., n = 6) of *Thais haemastoma* over time after direct transfer from 30 to 10‰ (○), and from 10 to 30‰ (●).

(Fig. 4) or from 30 to 10‰ (Fig. 5). After transfer from 10 to 30‰, activity of the snails remained low over the first 24 h. By day 3 all of the snails had reattached to the substrate indicating a normal activity pattern (Fig. 4A). Primary amine excretion fell during the 12 hours after the 10 to 30‰ transfer and slowly rose over the next 6 days. Amine loss fell to zero on day 10, and was not significantly different from control on day 14 (Fig. 4B). Ammonia excretion dropped precipitously immediately following the transfer and remained low for three days. Ammonia loss increased on days 4 and 5, and had returned to the control level by day 6 (Fig. 4C).

Snails remained unattached and withdrawn for the first 24 h after the 30–10‰ transfer (Fig. 5A). All had reattached by day 3 indicating a normal activity pattern (Fig. 5A). Amine excretion peaked on day 2 after transfer and returned to the control level by day 3 where it remained for the rest of the experiment (Fig. 5B). Ammonia excretion peaked on days 2–3 after transfer and remained fairly constant over days 4–7 before rising again by day 14 (Fig. 5C).

Ammonia loading

In snails acclimated to either 10 or 30‰, ammonia exchange with the medium was always in the direction of ammonia release, regardless of the amount of exogenous ammonia present. Twenty-four hours after snails were transferred from 10 to 30‰ there was a linear dose-related uptake of ammonia at exogenous ammonia concentrations up to 100 μM. At concentrations greater than 100 μM ammonia there was no change in the uptake rate (Fig. 6).

Over the first 24 hours of direct transfer from 10 to 30‰ the only amino acid to show any change in concentration was alanine (Table V), which, without exogenous ammonia in the high-salinity water, rose from 10 to 87 μmoles · g dry weight^{-1}. When either 175 or 350 μM ammonia was added to the high-salinity water, alanine levels rose at the same rate as in the control transfers for the first 12 h, but had leveled off

TABLE III

Free amino acid levels in foot tissue of Thais haemastoma *directly transferred from 10 to 30‰* [μ*moles · g dry weight*$^{-1}$ $\bar{x}$ *(±S.E.)* $n = 6$*, to nearest* μ*mole*]

Amino acid	Day 0	Day 1	%	Day 2	%	Day 3	%	Day 7	%	Day 10	%	Day 14	%
P-Serine	12 ± 2	13 ± 3	8	8 ± 0	−33	9 ± 1	−25	8 ± 1	−33	12 ± 1	0	9 ± 1	−25
Taurine	33 ± 3	32 ± 1	−3	30 ± 2	−9	32 ± 1	−3	33 ± 3	0	40 ± 4	21	28 ± 2	−15
Aspartate	35 ± 3	12 ± 3	−66	49 ± 3	40	49 ± 4	40	49 ± 2	40	59 ± 1	69	59 ± 2	69
Threonine	4 ± 1	4 ± 0	0	23 ± 3	475	25 ± 3	525	35 ± 4	775	26 ± 3	550	31 ± 4	675
Serine	5 ± 1	5 ± 1	0	26 ± 2	420	31 ± 3	520	39 ± 4	680	38 ± 4	660	43 ± 4	760
Glutamate	11 ± 1	16 ± 3	45	23 ± 0	109	21 ± 1	91	25 ± 2	127	26 ± 2	136	19 ± 0	73
Proline	N.D.	4 ± 0	—	18 ± 3	350[a]	28 ± 4	600[a]	34 ± 5	750[a]	38 ± 4	850[a]	40 ± 2	900[a]
Glycine	3 ± 1	7 ± 0	133	28 ± 2	833	47 ± 5	1463	70 ± 5	2233	72 ± 6	2300	86 ± 2	1627
Alanine	15 ± 1	68 ± 5	353	181 ± 13	1107	237 ± 19	1488	220 ± 27	1367	177 ± 6	1080	202 ± 12	1247
Arginine	37 ± 3	45 ± 3	22	45 ± 2	22	38 ± 1	3	30 ± 2	−19	33 ± 2	−11	36 ± 3	−3
Others	14	44	214	37	164	38	171	33	136	41	193	47	236
Total	169	249	48	468	177	555	228	576	241	562	233	600	255

[a] Based on Day 1.
% = % change.
N.D. = Not detected.

TABLE IV

Free amino acid levels in foot tissue of Thais haemastoma *directly transferred from 30 to 10‰ [μmoles · g dry weight^{-1} $\bar{x}$ ± (S.E.) n = 6, to nearest μmole]*

Amino acid	Day 0	Day 1	%	Day 2	%	Day 3	%	Day 7	%	Day 10	%	Day 14	%
P-Serine	6 ± 0	7 ± 1	17	8 ± 0	33	8 ± 1	33	10 ± 1	67	11 ± 1	83	12 ± 1	100
Taurine	82 ± 7	85 ± 3	4	88 ± 8	7	62 ± 2	−24	42 ± 2	−49	44 ± 3	−46	39 ± 2	−52
Aspartate	47 ± 4	41 ± 2	−13	50 ± 2	6	50 ± 3	6	36 ± 3	−23	41 ± 2	−12	39 ± 2	−17
Threonine	12 ± 2	13 ± 1	8	13 ± 2	8	8 ± 0	−33	3 ± 0	−75	3 ± 0	−75	2 ± 0	−83
Serine	20 ± 3	19 ± 1	5	24 ± 5	20	18 ± 3	−10	6 ± 0	−70	7 ± 1	−65	7 ± 1	−65
Glutamate	14 ± 1	20 ± 0	42	23 ± 1	64	23 ± 1	64	12 ± 1	−14	10 ± 2	−28	13 ± 1	−7
Proline	13 ± 2	21 ± 3	62	13 ± 3	0	5 ± 0	−61	N.D.	−100	N.D.	−100	N.D.	−100
Glycine	53 ± 8	72 ± 4	36	33 ± 9	−38	9 ± 2	−83	4 ± 0	−92	3 ± 0	−94	3 ± 0	−94
Alanine	42 ± 4	86 ± 6	105	38 ± 9	−10	24 ± 4	−43	10 ± 1	−76	9 ± 1	−79	12 ± 2	−71
Arginine	25 ± 2	28 ± 1	12	31 ± 1	24	32 ± 2	28	25 ± 2	0	36 ± 1	44	34 ± 2	36
Others	36	44	22	22	−39	31	−14	45	25	51	42	40	11
Total	350	436	25	343	−2	270	−23	193	−45	215	−39	201	−42

% = % change.
N.D. = Not detected.

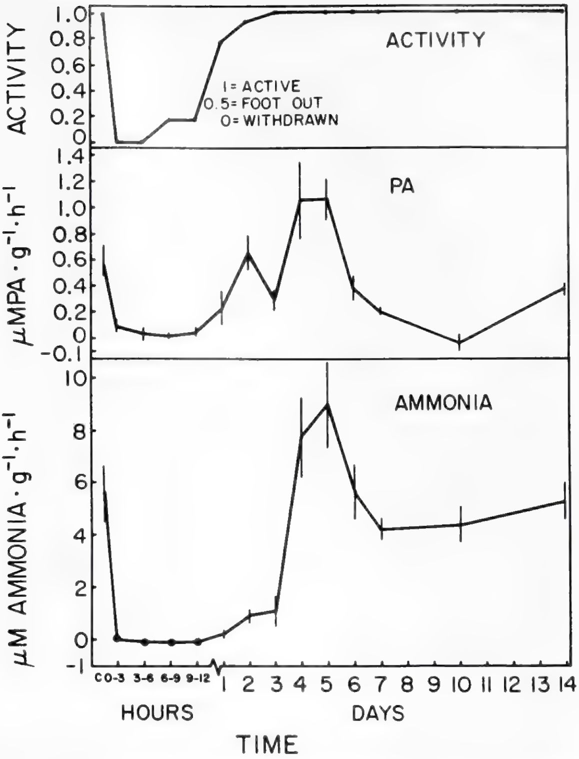

FIGURE 4. Activity (A, 0 = operculum closed, 0.5 = foot out, but not attached, 1.0 = foot out and attached to substrate), ammonia excretion (B, in μmoles · g dry weight^{-1} · h^{-1}) and primary amine excretion (C, in μmoles · g dry weight^{-1} · h^{-1}) over 14 days in *Thais haemastoma* directly transferred from 10 to 30‰. ($\bar{x}$ ± S.E., n = 12).

by hour 24. The total FAA pool at 24 h after transfer is not significantly different in the NH_4^+-spiked transfers and in the control transfer due mainly to the very large variation in alanine concentration at 24 h in the control transfer (86.5 ± 15.8 μmoles · g dry weight^{-1}—Table V).

DISCUSSION

Thais haemastoma is a euryhaline species that partially regulates its volume by changes in the intracellular free amino acid pool. The FAA pool of *T. haemastoma* is composed of a mixture of seven quantitatively important free amino acids (Table I) rather than being dominated by a single free amino acid, such as taurine, as occurs in *T. lapillus* (Stickle *et al.*, in press). When subjected to altered salinity, volume regulation is achieved by the rapid initial alteration of the intracellular concentrations of alanine and glycine.

The water content of *Thais haemastoma* remained remarkably constant across acclimation salinities (Table II), and had returned to the steady state level two weeks after a direct salinity transfer in either direction between 30 and 10‰ (Fig. 3). Since the analysis of variance for water content *versus* salinity was not significant over the range of 5 to 30‰, we must assign a value of zero to Oglesby's (1975) beta. Hildreth

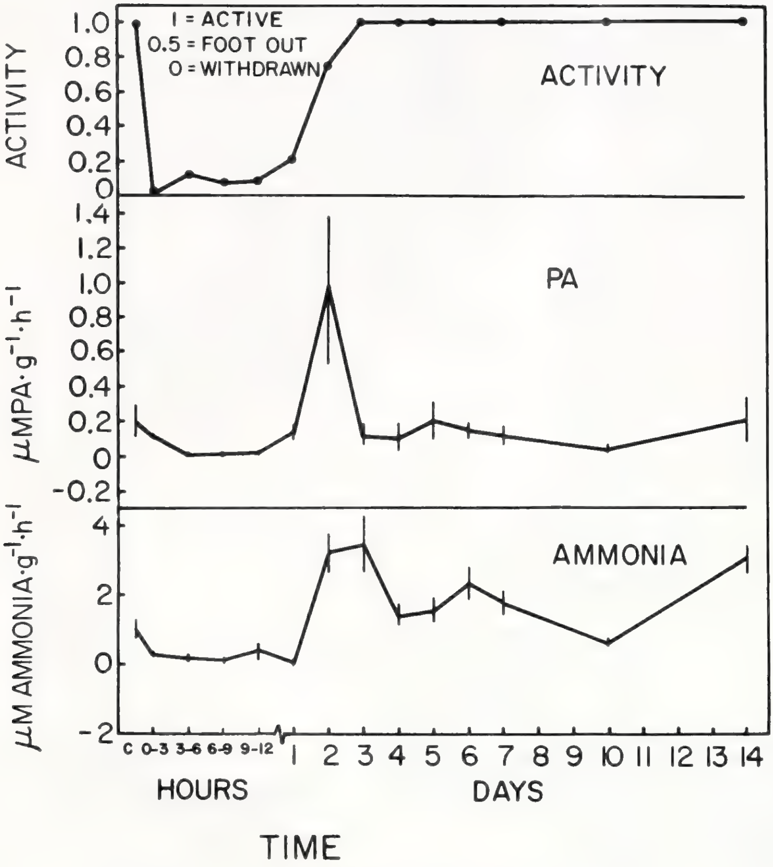

FIGURE 5. Activity (A, 0 = operculum closed, 0.5 = foot out, but not attached, 1.0 = foot out and attached to substrate), ammonia excretion (B, in μmoles · g dry weight^{-1} · h^{-1}) and primary amine excretion (C, in μmoles · g dry weight^{-1} · h^{-1}) over 14 days in *Thais haemastoma* directly transferred from 30 to 10‰. ($\bar{x}$ ± S.E., n = 12).

and Stickle (1980) found a very small, yet statistically significant increase in body water content in *T. haemastoma* as the acclimation salinity was decreased from 30 to 10‰ from 72% to 77%, 10‰ yielding a beta of 0.07, still indicative of excellent volume regulation. This is in direct contrast to the pattern of body water regulation in *Thais lapillus,* where beta values range from 0.22 to 1.26, depending on the temperature (Stickle *et al.,* in press).

Although there is no significant change in the total amount of body water in snails acclimated to salinities between 5 and 30‰ and the hemolymph of *T. haemastoma* is isosmotic to ambient seawater (Stickle and Howey, 1975; Hildreth and Stickle, 1980), it is possible that the distribution of water between the intra- and extracellular compartments might show a reciprocal change with salinity. Staaland (1970) was able to measure the volume of the extracellular space (as inulin space) in *Buccinum undatum* acclimated to several salinities and found that as salinity was raised from 10 to 35‰, the size of the intracellular fluid compartment decreased as the size of the extracellular compartment increased. The total amount of body water decreased and the concen-

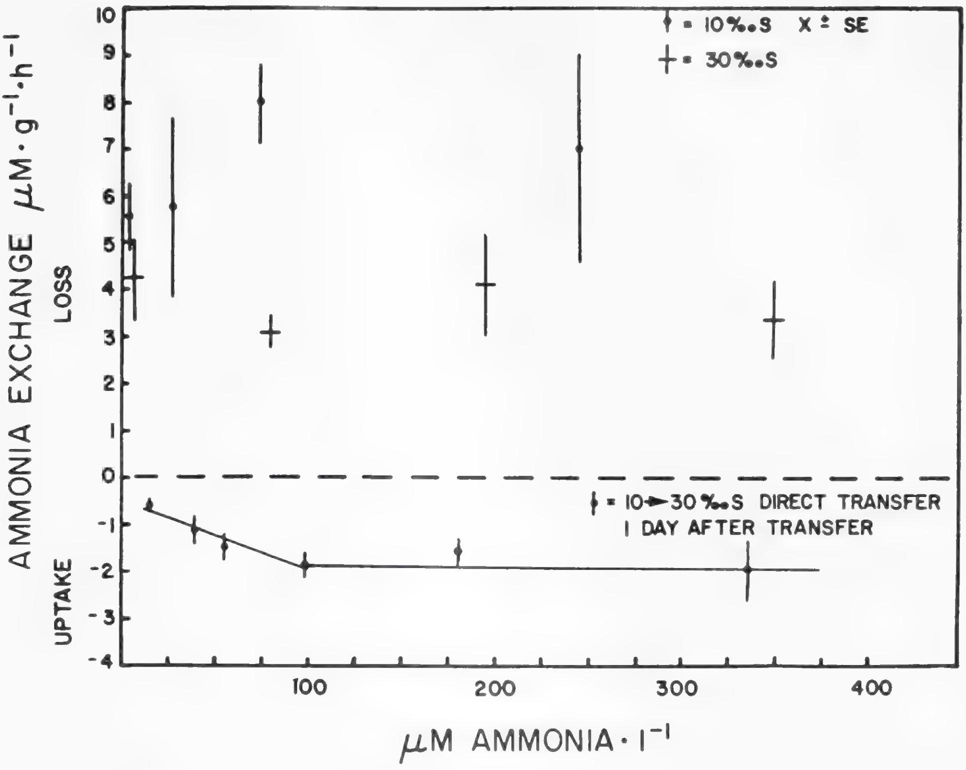

FIGURE 6. Ammonia excretion rates of *Thais haemastoma* (μmoles · g dry weight^{-1} · h^{-1}, $\bar{x} \pm$ S.E., n = 12) as a function of exogenous ammonia in the medium.

tration of intracellular NPS increased with the increasing salinities. Since the total body water in *T. haemastoma* remains constant between 5 and 30‰, it might be argued that changes in the FAA pool are only reflections of a constant amount of FAA being diluted or concentrated by varying amounts of intracellular water, and this would appear to be the case when FAA levels are expressed in terms of cellular hydration. Although we did not use an ECF marker in this study, it is reasonable to assume that as the acclimation salinity decreased the size of the intracellular compartment increased at the expense of the extracellular compartment. Indirect evidence for such a change is given by Findley *et al.* (1978) who report increasing difficulty in obtaining hemolymph samples from *T. haemastoma* as the exposure salinity decreased. Expressed on a dry weight basis, the increase in cellular FAA in *T. haemastoma* is real, indicating that these solute molecules are being used as osmotic effectors.

The percent body water in the snails used in steady-state salinity experiments was higher at both 10 and 30‰ than in those snails used in the direct transfer experiments, nearly a 10% difference at 10‰. The steady-state salinity experiments were done using snails collected in mid spring, and the direct transfer experiments were done using snails collected in mid-summer, before and after the breeding and capsule deposition season for this population of southern oyster drills (pers. obs.). Stickle (1973) found a seasonal variation in the body water index (g body water · g live weight^{-1} · 100) in a population of *T. lamellosa* acclimated to 30‰ from near Friday Harbor, Washington.

TABLE V

Free amino acid levels in the foot of Thais haemastoma *after direct transfer from 10 to 30‰ under conditions of ammonia loading [μmoles · g dry weight*$^{-1}$ $\bar{x} \pm$ *(S.E.) n = 6, to nearest μmole]*

Amino acid	10‰	+0 μM				+175 μM				+350 μm			
		12 h	%	24 h	%	12 h	%	24 h	%	12 h	%	24 h	%
P-Serine	14 ± 0	13 ± 1	−7	12 ± 1	−14	13 ± 0	−7	15 ± 1	7	16 ± 2	14	14 ± 0	0
Taurine	24 ± 1	23 ± 2	−4	30 ± 3	25	23 ± 1	−4	25 ± 1	4	24 ± 0	0	28 ± 1	17
Aspartate	38 ± 1	6 ± 1	−84	32 ± 4	−16	10 ± 1	−74	5 ± 1	−87	5 ± 0	−87	17 ± 4	−55
Threonine	2 ± 0	2 ± 0	0	5 ± 0	160	1 ± 0	−50	1 ± 0	−50	2 ± 0	0	2 ± 0	0
Serine	5 ± 0	3 ± 0	−40	10 ± 2	100	3 ± 0	−40	3 ± 0	−40	3 ± 0	−40	3 ± 0	−40
Glutamate	11 ± 0	14 ± 0	27	18 ± 0	64	15 ± 1	36	9 ± 1	−18	12 ± 1	9	14 ± 0	27
Proline	N.D. —	4 ± 1	—	7 ± 0	75[a]	N.D. —	—	N.D. —	—	N.D. —	—	4 ± 1	0[a]
Glycine	4 ± 1	4 ± 0	0	7 ± 1	75	4 ± 1	0	3 ± 0	−25	3 ± 0	−25	6 ± 1	50
Alanine	10 ± 1	43 ± 5	330	86 ± 16	760	40 ± 3	300	48 ± 6	380	38 ± 3	280	51 ± 5	410
Arginine	47 ± 2	41 ± 2	−13	48 ± 1	2	50 ± 2	6	37 ± 4	−21	35 ± 1	−25	42 ± 1	−11
Others	8	18	11	25	212	14	75	13	62	13	62	20	150
Total	163	171	5	280	72	173	6	159	−2	151	−7	201	23

[a] Based on 0 μM 12 h.
% = % change.
N.D. = Not detected.

Giese (1969) found an inverse relationship between body water and lipid content in pre-spawning black abalone, *Haliotis cracheroidii,* and Belisle and Stickle (1978) suggest that seasonal changes in lipid concentration may mask a relationship between percent body water and salinity in *T. haemastoma.*

The free amino acid pool of *T. haemastoma* acclimated to steady state salinities is not dominated by any one amino acid; rather a combination of alanine, glycine, glutamate, aspartate, arginine, proline, and taurine combine to constitute up to 92% of the intracellular free amino acid pool. Of these, alanine and glycine are the most common, comprising up to 25% each of the FAA pool at 15‰. Alanine predominates in the FAA pool during high-salinity acclimation.

A single amino acid is often dominant in the FAA pool of marine invertebrates. Taurine dominates the FAA pool of many stenohaline gastropod species, making up approximately 75% of the FAA pool in a population of *Thais lapillus* from England (Stickle *et al.,* in press), and 52% in a population from France (Hoyeaux *et al.,* 1976). Similarly, taurine accounted for 38% of the FAA in *Littorina littorea,* 75% in *Patella vulgata* (Hoyeaux *et al.,* 1976), and 78% in *Thais emarginata* (Emerson, 1969). Glycine or alanine are often predominant in the intracellular FAA pool of euryhaline species, making up to 53% of the FAA in *Mytilus edulis,* 33% in *Scrobicularia plana* (Hoyeaux *et al.,* 1976), 33–75% in *Rangia cuneata* (Fyhn, 1976; Henry *et al.,* 1980), 45% in *Crangon crangon* (Weber and van Marrewijk, 1972), and 25% in *Thais haemastoma* (current study).

Taurine metabolism is poorly understood, but it appears that it is very slowly formed and catabolized in molluscs (Bishop *et al.,* 1983). Taurine is certainly being used as an osmotic effector in *T. haemastoma,* comprising 14% of the FAA pool at 20‰ (Table I). Taurine is not being formed in the cells during the first two weeks of the 10 to 30‰ transfer, its concentration actually decreasing slightly (Table III). Over the course of the 30 to 10‰ transfer, taurine behaves like the other labile amino acids, slowly leaving the cells with time after the salinity decrease (Table IV). Taurine comprises 75% of the FAA pool in *T. lapillus,* and its concentration remains constant at 300 μmoles · g dry weight^{-1} over the range of acclimation salinities between 17.5 and 35‰ (Stickle *et al.,* in press). It has been hypothesized that the low salinity tolerance of *T. lapillus* is due to the fact that taurine makes up such a large percentage of the FAA, leaving no sizable pool of mobile FAA available for adjustment of the intracellular osmotic pressure (Stickle *et al.,* in press).

In isolated ventricles of the ribbed mussel, *Modiolus demissus,* transferred from 12 to 36‰, osmoregulation was virtually complete within five days after the salinity transfer (Baginski and Pierce, 1975). The alanine concentration rose immediately after transfer and started to decline after eight days by which time glycine levels had started to rise. The taurine concentration, which had been similar to that of both alanine and glycine at 16‰ showed a very slow rise over the course of the 101 day experiment until its concentration was once again equal to alanine and glycine; each amino acid contributing about one third of the total free amino acid pool (Baginski and Pierce, 1975). Free alanine and glycine are clearly being used as immediate osmotic effectors, taurine apparently only contributing more as an osmotic effector during very long-term osmotic adaptation to high salinity.

Although we only followed our transfer experiments for 14 days, an immediate increase in the concentrations of alanine and glycine similar to that found in *M. demissus* (Baginski and Pierce, 1975) was seen during high-salinity adaptation in *T. haemastoma.* By the second day after the 10 to 30‰ transfer the concentrations of these two amino acids exceeded their concentrations in animals acclimated to 30‰ (Tables I, III). The tendency for alanine concentration to slowly decline after day 3 of the hyperosmotic transfer (Table III) suggests that true return of the FAA pool to steady state conditions in *T. haemastoma* takes much longer than two weeks following a transfer to higher salinity.

Alanine and glycine were also the most labile members of the free amino acid pool even in the hyposmotic transfer (Table IV). Concentrations of both alanine and glycine rose over the first 24 h of the 30 to 10‰ transfer; the opposite of the expected response. This increase in glycine and alanine levels took place during the time when the operculum was tightly closed in all individuals, with consequent isolation from the environment and probable anaerobic metabolism. The transient increase in intracellular glycine and alanine (Table III) over the first two days of the 30 to 10‰ transfer might be attributable to anaerobic metabolism. Alanine has been found to accumulate in *T. haemastoma* after 24 h in nitrogen-saturated water (Ellington, pers. comm.). When subjected to a diurnal salinity fluctuation cycle of either 10–30–10 or 30–10–30‰ the oxygen consumption rate of *T. haemastoma* dropped off markedly and the siphon was retracted during the course of the salinity change (Findley *et al.,* 1978).

The only essential free amino acid found in appreciable quantities in foot tissue of *Thais haemastoma* was arginine. Its concentration remained remarkably constant over the entire range of acclimation salinities tested (Table I). Even after a direct transfer from high to low or from low to high salinity the arginine concentration did not change (Tables III, IV). Somero and Bowlus (1983) noted that at physiological pH's, the guanidino group of arginine carries a positive charge, leading to a disruption of enzyme function at elevated levels of free arginine. It seems reasonable then that free arginine concentration be strictly controlled, especially since the phosphogen in molluscs is arginine phosphate. Somero and Bowlus (1983) suggested that one way arginine levels are controlled in molluscan tissue during periods of high metabolic demand is the formation of octopine from pyruvate and arginine. Octopine does not affect enzyme structure or function at physiological levels. Livingstone *et al.* (1983) have found significant levels of octopine dehydrogenase in *Thais (=Nucella) lapillus, Buccinum undatum,* and *Neptunea antiqua.* We have only found trace levels of octopine dehydrogenase activity in foot tissue of *Thais haemastoma* (Kapper and Stickle, unpubl.). If changes in free arginine concentrations are disruptive to metabolism in *T. haemastoma,* arginine concentration is regulated in some other way.

After a decrease in ambient salinity, cell volume is initially increased by the osmotic influx of water. Volume is restored by the expulsion of solute from the cells along with osmotically obligated water (Pierce and Amende, 1981). The fate of amines lost from the FAA pool during low salinity adaptation could be excretion, deamination, or transamination followed by excretion (Bishop, 1976). If amines are lost from the cells during low salinity acclimation, then one would expect to see at least a transient increase in the level of free amino acids in the hemolymph. Stickle and Howey (1975) found the hemolymph NPS of *T. haemastoma* to increase during the low salinity phase of a 24-hour 30–10–30‰ salinity fluctuation. Similarly, Livingstone *et al.* (1979), noted an slight increase in hemolymph amino acids and ammonia in *Mytilus edulis* when transferred from 30 to 15‰, as did Strange and Crowe (1979) *in Modiolus demissus.*

If free amino acids are released from the cells and deaminated during low salinity adaptation and the resulting amino group excreted, there would be a pulse of ammonia excretion soon after hyposmotic transfer. Lange (1964) termed this efflux of free amino acids or ammonia the regulatory step for volume regulation. There was a peak in ammonia and primary amine excretion on days two and three after transfer from 30 to 10‰ in *T. haemastoma* (Fig. 5).

There was also a peak in ammonia and free amino acid excretion during the second and third days of the 10 to 30‰ transfer (Fig. 4). The periods of increased nitrogen excretion after transfer occurred after the snails had begun to reopen their opercula and expose the foot to the external environment. It can be hypothesized that the animals are anaerobic during the time that their opercula are closed, and that nitrogenous metabolites are accumulating in the tissues. If the total amount of nitrogen excreted during the time the snails are isolated is compared to the total amount of excreta expected had closure not occurred, there is a deficit of nearly 380 μmoles · g^{-1} over the three day period. During the second three days of the experiment, while excretory rates were elevated, the total amount of nitrogen excreted was 95 μmoles · g^{-1} greater than would be expected had closure not occurred. This does not account for all of the deficit of the first two days, but if the snails are using primarily anaerobic pathways during this time, then it is reasonable to assume that the overall magnitude of metabolism and thus production of ammonia and primary amines is reduced (Gade, 1983). The excretory pulse after reopening then probably does represent a flushing of accumulated metabolites from the body.

The data regarding the use of exogenous ammonia as an aminating source during high-salinity adaptation are not conclusive. Snails acclimated to steady state salinities do not take up ammonia from the ambient water even at concentrations up to 350 μM. When exogenous ammonia is available during the course of high salinity acclimation, it is taken into the animal, apparently in some saturable fashion (Fig. 6). The fate of the ammonia taken up by the animal cannot be determined without ^{15}-N tracer experiments.

To summarize, *Thais haemastoma* partially adapts to increased or decreased salinities by changing the size of the intracellular free amino acid pool. During an increase in ambient salinity, increases in alanine and glycine concentrations account for much of the increased intracellular osmolality. Volume regulation, as evidenced by changes in the amount of total body water is mostly complete within three days of the transfer, as are the largest changes in cellular free amino acid concentrations. Longer than 14 days are required for the intracellular free amino acid profile to return to the steady state pattern after hyperosmotic transfer. After a decrease in ambient salinity, alanine, glycine, and taurine are lost from the cells, and volume regulation is complete by three days after the transfer. It is clear that changes in the concentrations of intracellular free amino acids contribute significantly to the salinity adaptation process in *Thais haemastoma,* but these changes are probably not the only mechanism used by this species to cope with altered salinity.

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MULTIPLE VARIANTS OF MYOFIBRILLAR PROTEINS IN SINGLE FIBERS OF LOBSTER CLAW MUSCLES: EVIDENCE FOR TWO TYPES OF SLOW FIBERS IN THE CUTTER CLOSER MUSCLE[1]

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ABSTRACT

SDS-polyacrylamide gel electrophoresis of myofibrillar proteins in single fibers of lobster claw closer muscles distinguished three types of fibers: one fast and two slow. The fibers differed both qualitatively and quantitatively in the variants of paramyosin and troponin present. There were four proteins unique to fast fibers (paramyosin$_1$, a 75-kD protein, troponin-I$_3$, and -I$_5$) and two proteins unique to slow fibers (troponin-I$_4$ and -C$_1$). Fast fibers were found only in the cutter claw. The major type of slow fibers (S$_1$) appeared to account for the entire muscle mass in the crusher claw as well as ~85% of the slow fibers in the cutter claw. Another type (S$_2$) comprised 10–15% of the slow fibers in the cutter claw. The S$_1$ and S$_2$ fibers differed in the variants of troponin-I and -T. The S$_2$ fibers contained troponin-T$_1$ and I$_2$ as the major variant of troponin-I; S$_1$ fibers lacked T$_1$ and contained I$_4$ as the major isoform. These data indicate that the heterogeneity of myofibrillar proteins observed in actomyosins extracted from whole muscle (Mykles, 1985) is due to three populations of fibers, each containing its own assemblage of regulatory and contractile isoforms. More than one variant of a myofibrillar protein can be expressed in a single fiber, forming unique assemblages by which subgroups can be discriminated within the broader categories of fast and slow fibers.

INTRODUCTION

Physiological, morphological, and histochemical methods have been used to classify crustacean muscle fibers. Fast, or phasic, fibers have short sarcomeres, low ratios of thin:thick myofilaments, fast contraction speeds, low oxidative capacities, and high ATPase activities. Conversely, slow, or tonic, fibers have longer sarcomeres, higher ratios of thin:thick myofilaments, slower contraction speeds, higher oxidative capacities, and lower ATPase activities (Jahromi and Atwood, 1969, 1971; Lang *et al.,* 1977, 1980; Mykles and Skinner, 1981; Stephens *et al.,* 1984; Mykles, 1985). Fibers with intermediate properties have also been described (Jahromi and Atwood, 1971; see review by Govind and Atwood, 1982). Occasionally these methods can discriminate between fiber subgroups (Kent and Govind, 1981; Parsons and Mosse, 1982). More often characteristics defined by such methods do not delineate absolute differences between fast, slow, and intermediate fibers within a species; comparisons between species are even less definitive. SDS-polyacrylamide gel electrophoresis is a method that should serve to more rigorously distinguish muscle fiber types in Crustacea.

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[1] Abbreviations used: ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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Heterogeneity of myofibrillar proteins has been described recently in the muscles of two crustacean species, the lobster, *Homarus americanus* (Costello and Govind, 1984; Mykles, 1985), and snapping shrimp, *Alpheus heterochelis* (Quigley and Mellon, 1984). Moreover, actomyosins extracted from lobster muscles contain multiple variants of regulatory and contractile proteins (Mykles, 1985). These isoforms of myofibrillar proteins comprise four distinct assemblages in fast and slow muscles. A total of two variants of paramyosin, three of troponin-T, five of troponin-I, three of troponin-C, three of myosin alpha light chain (αLC) and two of myosin beta light chain (βLC) are found in six muscles of the claws and abdomen (Mykles, 1985). Myosin heavy chain, actin, and tropomyosin appear isomorphic in all lobster muscles examined. The closer muscles of the cutter and crusher claws differ primarily in the species of paramyosin and troponin-I and -C; two or more variants of these proteins can occur in a single muscle. This heterogeneity may be caused by mixtures of different fibers, each having particular protein variants, or by a homogeneous population of fibers that contain a mixture of protein variants in a proportion characteristic of the specific muscle. To distinguish between these two alternatives, individual fibers from the claw closer muscles of adult lobsters were glycerinated and analyzed by SDS-PAGE which, coupled with silver staining, is sufficiently sensitive to detect proteins from single fibers. The data show three distinct fiber types, rather than the two types classically defined (see Govind and Atwood, 1982, for references). The slow fibers of the crusher claw and

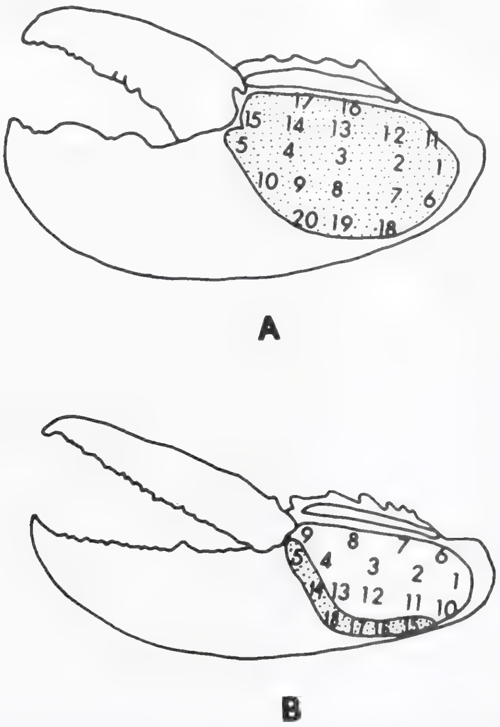
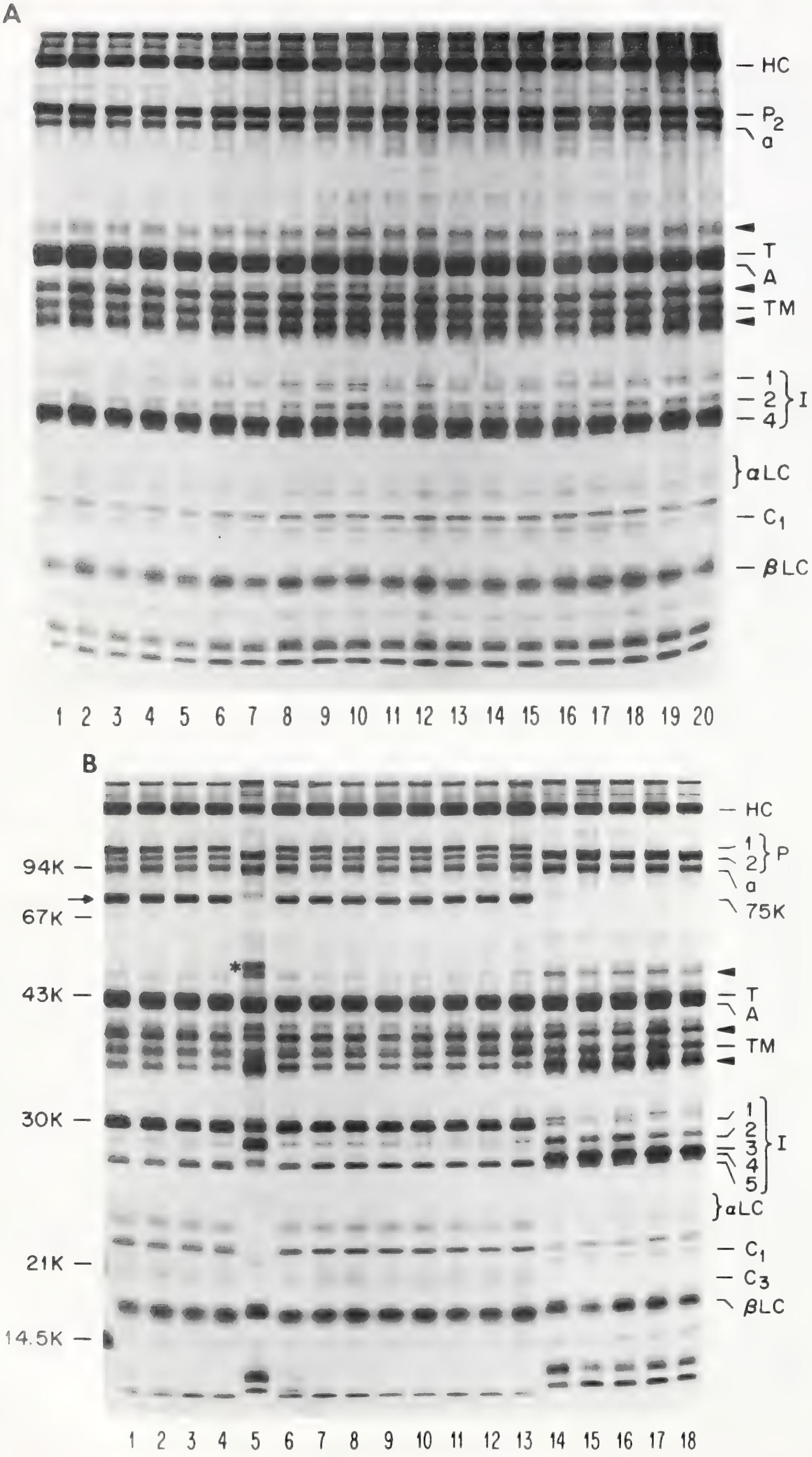


FIGURE 1. Diagram of lobster crusher (A) and cutter (B) claws showing locations from which single fibers were removed for analysis by SDS-PAGE. Numbers correspond to lane numbers in Figure 2. Stippling indicates areas containing slow fibers; fast fibers were located in the dorsal and proximal regions of the cutter closer muscle (see Lang *et al.*, 1980).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the fast fibers of the cutter claw consisted of homogeneous populations. By contrast, there were two types of slow fibers in the cutter claw, each containing a different assemblage of troponin variants.

MATERIALS AND METHODS

Homarus americanus (0.6–0.9 kg) were obtained locally. Claws were removed and placed on ice for 4 h, during which time the epidermis partially separated from the exoskeleton, facilitating removal of the muscle; this phenomenon has been termed "forced apolysis" (O'Brien *et al.*, 1984). Tissues were rinsed twice in cold 0.5 *M* NaCl and 5 m*M* sodium phosphate (pH 7.4) and glycerinated in cold buffer containing 20 m*M* Tris-acetate (pH 7.5), 50% glycerol, 0.1 *M* KCl, 1 m*M* EDTA, 0.1% Triton X-100 for 2–3 h. Although fibers vary in diameter, this procedure was sufficient to extract most of the soluble proteins. Single fibers were removed from the muscle (Fig. 1) and solubilized in 0.25 or 0.5 ml SDS sample buffer [62.5 m*M* Tris-HCl (pH 6.8), 12.5% glycerol, 1.25% SDS, 1.25% β-mercaptoethanol] overnight at room temperature. Samples were heated at 90°C, 3 min before electrophoresis. Glycerination was kept to a minimum since fibers extracted for longer periods (>12 h) were not completely solubilized in SDS sample buffer.

SDS-PAGE was done using a discontinuous gel system as described (Mykles and Skinner, 1982, 1983; Mykles, 1985). BioRad low molecular weight standards were used. Gels were fixed in 10% glutaraldehyde (Schleicher and Watterson, 1983) and stained with silver (Wray *et al.*, 1981). Protein concentrations were determined by fluorescence emission spectroscopy (Mykles and Skinner, 1982).

RESULTS AND DISCUSSION

The claw muscles of the American lobster, *Homarus americanus*, contain numerous variants of myofibrillar proteins (Mykles, 1985). These comprise two isoforms of paramyosin (P_1, $M_r = 110{,}000$; P_2, $M_r = 105{,}000$), three of troponin-T (T_1, $M_r = 55{,}000$; T_2 and T_3, $M_r \sim 48{,}000$), five of troponin-I (I_1, $M_r = 30{,}000$; I_2, $M_r = 29{,}000$; I_3, $M_r = 28{,}500$; I_4, $M_r = 28{,}000$; I_5, $M_r = 27{,}000$), two of troponin-C (C_1, $M_r = 20{,}000$; C_3, $M_r = 17{,}500$), three of myosin alpha light chain (αLC_1, $M_r = 23{,}500$; $\alpha LC_2 = 22{,}000$; $\alpha LC_3 = 21{,}000$), and one of myosin beta light chain ($M_r = 18{,}500$). The troponin-T variants T_2 and T_3 were poorly resolved on 10% polyacrylamide gels and were usually obscured by the large amount of actin ($M_r = 42{,}000$) present (Fig. 2). Neither troponin-C nor myosin αLC are stained intensely with Coomassie blue or silver; gels must be overloaded (Mykles, 1985). Gels were fixed with glutaraldehyde (Schleicher and Watterson, 1983) before silver-staining to enhance visualization of both proteins (Fig. 2).

FIGURE 2. SDS-polyacrylamide gels of glycerinated single fibers from lobster crusher (A) and cutter (B) claw closer muscles. Lane numbers correspond to locations diagrammed in Figure 1. Myofibrillar proteins are actin (*A*), myosin heavy (*HC*) and light (*LC*) chains, α-actinin (*a*), paramyosin (*P*), troponin-T (*T*), -C (*C*), and -I (*I*), and tropomyosin (*TM*). Crusher claw (A, all lanes) contained a uniform population of slow (S_1) fibers; I_4 was the major variant of troponin-I. Fast fibers (B, lanes 1–4, 6–13) contained a 75-kD protein (arrow) absent from slow fibers (B, lanes 5, 14–18). A small proportion (10–15%) of slow fibers (S_2) in the cutter claw contained variant T_1 of troponin-T (asterisk) and possessed I_2 as the major species of troponin-I (B, lane 5). Three major unidentified proteins (arrowheads) occurred in both fast and slow fibers. In S_1 fibers a minor protein slightly smaller than troponin-I_4 (A, all lanes; B, lanes 14–18) could be confused with the troponin-I_5 of fast fibers (B, lanes 1–4, 6–13); the troponin variants and the minor protein had distinct electrophoretic mobilities. Each lane contained 10 μg protein. Positions of molecular weight standards are indicated at left of (B).

In preparations from whole muscle, the closer muscles of the cutter and crusher claws differed primarily in the variants of paramyosin and troponin-I and -C (Mykles, 1985). Not surprisingly, a similar dichotomy was seen in individual fibers. Slow fibers in both claws contained one species of paramyosin (P_2), three of troponin-I, (I_1, I_2, and I_4), and one of troponin-C (C_1) (Fig. 2A, all lanes; B, lanes 5, 14–18). Individual fast fibers contained two variants of paramyosin (P_1, P_2), four of troponin-I (I_1, I_2, I_3 and I_5), and one of troponin-C (C_3) (Fig. 2B, lanes 1–4, 6–13). Slow fibers contained two small proteins (<14.5 kD) that were absent from fast fibers. In addition, fast fibers contained a 75-kD protein that was absent from slow fibers. This protein appears to be a component of the myofibrillar apparatus of fast fibers; it occurs in actomyosins extracted from cutter-claw closer and deep abdominal flexor and extensor muscles (Costello and Govind, 1984; Mykles, 1985).

Analysis of myofibrillar proteins of individual fibers has shown that the closer muscle of the crusher claw consisted entirely of slow fibers (Fig. 1A), while that of the cutter claw contained primarily fast fibers with a small population of slow fibers in the ventral and distal portions (Fig. 1B). These distributions agreed well with those obtained by morphological and histochemical methods (Lang *et al.*, 1977, 1980).

All the fibers possessed three prominent proteins (Fig. 2A, B, arrowheads; M_r = 35,000; 38,000; 49,000) that are not found in actomyosins extracted from muscle homogenates with high salt (Mykles, 1985; compare also lane *a* with lanes *b*, *c* in Fig. 3 of this paper). Since glycerination should extract all soluble proteins from the fiber, it seems likely that these are cytoskeletal elements that were not solubilized in 0.6 *M* NaCl.

It seemed possible that the various species of proteins, particularly troponin-I and paramyosin, occurred in distinct subgroups of slow and fast fibers; analysis of single fibers showed that more than one variant of a myofibrillar protein occurred in individual fibers (Fig. 2A, B). Thus the fast fibers in the cutter claw and the slow fibers in the crusher claw each consisted of a homogeneous population of fibers containing the same assemblage of myofibrillar protein variants; there were no apparent differences, either qualitative or quantitative, in the distribution of these variants.

The pattern in the slow fibers of the cutter claw, however, was more complex; two fiber types contained distinct assemblages of protein variants. Both types were slow fibers: they contained P_2 as the only species of paramyosin and they lacked the 75-kD protein characteristic of fast fibers (Fig. 2B). The majority of the slow fibers, termed S_1, had a pattern identical to that of crusher closer fibers, which contained I_4 as the major species of troponin-I (compare Fig. 2A, all lanes, with Fig. 2B, lanes 14–18). However, the minor type (S_2), comprising 10–15% of the slow fibers in the cutter claw, contained I_2 as the major variant of troponin-I and troponin-T_1, which was absent from S_1 fibers (Fig. 2B, lane 5; see also Mykles, 1985). Thus S_1 and S_2 differed both qualitatively and quantitatively only with respect to troponin variants; other myofibrillar protein variants were shared by both.

Four distinct protein assemblages have been described in lobster claw and abdominal muscles; these patterns have been classified as cutter-claw closer, crusher-claw closer, deep abdominals, and superficial abdominals (Mykles, 1985). The variant pattern of the S_2 fibers of the cutter closer is potentially a fifth assemblage. However, comparison of the S_2 assemblage with that of the superficial abdominals showed that they were similar: both shared the same myofibrillar protein variants including troponin-T_1 and I_2 as the major isoform of troponin-I (Fig. 3, compare lanes *a* and *b*). I_4, the major variant in S_1 fibers, was present in small amounts in S_2 fibers and superficial abdominal actomyosins (Fig. 3; see also Mykles, 1985). Therefore the S_2 pattern is not a new assemblage. Rather, the S_2 fibers and superficial abdominal fibers are the same fiber type with possibly similar contractile properties.

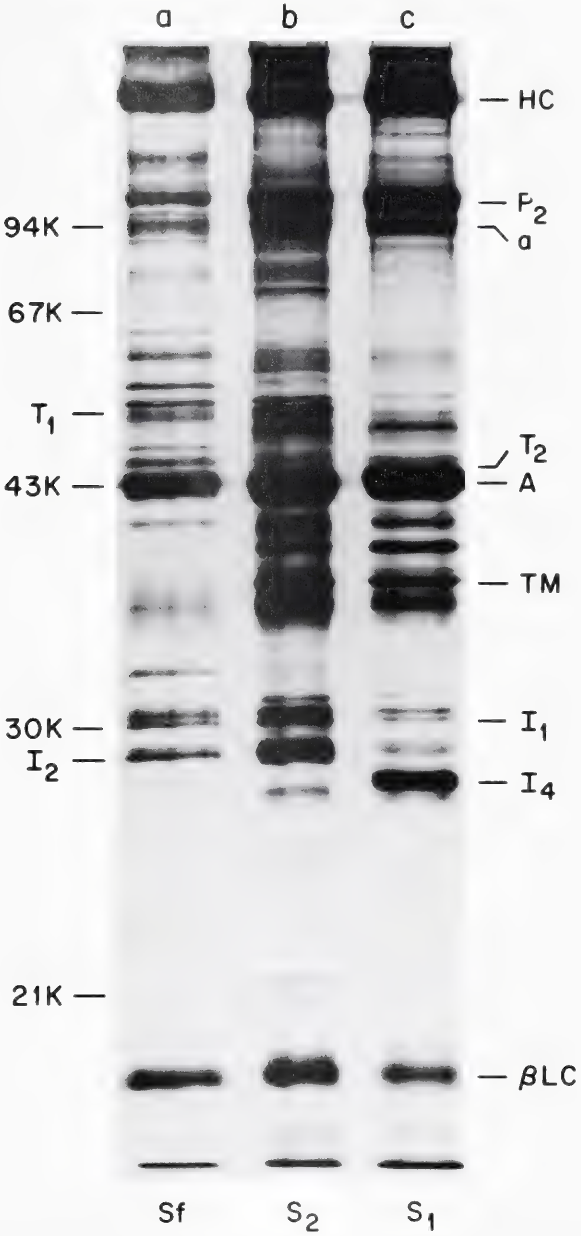

FIGURE 3. SDS-polyacrylamide gel of the two different types of slow fiber assemblages in actomyosin extracted from superficial abdominal flexor muscle (Sf, lane *a;* see Mykles, 1985) and myofibrillar proteins of glycerinated fibers from cutter closer muscle (lanes *b, c*). The minor type (S_2) contained a variant pattern similar to Sf muscle; both possessed troponin-T_1 and I_2 as the major variant of troponin-I. The major type (S_1) lacked T_1 and contained I_4 as the major isoform of troponin-I. Tropomyosin was originally present in Sf actomyosin (Mykles, 1985) but upon long-term (>1 year) storage in SDS sample buffer at −60°C it was not detected in later gels (lane *a*). See legend to Figure 2 for identity of myofibrillar proteins. Lane *a* contained 15 μg protein and lanes *b* and *c* each contained 20 μg protein. Positions of molecular weight standards are indicated at left.

Using histochemical methods, Kent and Govind (1981) demonstrated two types of slow fibers in the opener and closer muscles of lobster claws. The majority of the slow fibers in both claws have low ATPase and high NADH diaphorase activities, indicative of their slow contraction times and high oxidative capacity. In addition, a small group of tonic fibers found in a mid-lateral band in the distal regions of the closer muscles and in the proximal regions of the opener muscles of both claws have even lower ATPase and higher NADH diaphorase activities than the majority of slow fibers, suggesting that their contractile speeds are even slower and that the fibers are less easily fatigued.

It is not known whether the S_2 fibers described here are the same as the minor tonic fibers described by Kent and Govind. In this study the S_2 type was also found in the medial-distal region of the cutter closer. However, no S_2 fibers were found in the crusher closer even though the medial-distal region was sampled (Fig. 1A). Given the limited sampling from glycerinated muscle and the low frequency of these fibers, it is possible that the S_2 fibers were not detected. There were no apparent differences in morphology between S_1 and S_2 fibers which could be distinguished only by SDS-PAGE. An S_2 fiber was found in the ventral-proximal region of the cutter closer muscle, an area apparently lacking the lower ATPase/higher NADH diaphorase type (Kent and Govind, 1981). If the S_2 and minor tonic fibers are the same then their different contractile properties, as reflected by ATPase activity, may be related to different variants of the regulatory protein troponin or possible differences in actin and myosin variants that single-dimension gels may not detect.

Additional evidence supporting the identity of S_2 and minor tonic fibers is the similarity in the S_2 proteins to the proteins recovered in the actomyosin extracted from superficial abdominal muscles. Since the latter muscles are involved in slow movements and posture of the abdomen (Govind and Atwood, 1982), their fibers, like the minor tonic fibers, would be expected to have slow contractile speeds and to be very resistant to fatigue. It would not be surprising if they shared biochemical properties as well as myofibrillar protein variants with the S_2 fibers.

Crustacean muscle fibers have usually been characterized on the basis of physiological, histochemical, and morphological criteria. Although these methods can often discriminate between fast and slow fiber types and occasionally distinguish subtypes (Kent and Govind, 1981; Parsons and Mosse, 1982), the considerable variability makes it difficult to classify intermediate fibers. It is apparent that SDS-PAGE provides another tool for characterizing fiber types. SDS-PAGE demonstrated four polypeptides unique to fast fibers (P_1, a 75-kD protein, troponin-I_3, and -I_5) and two polypeptides unique to slow-fibers (troponin-I_4 and -C_1). The distinct variant assemblages revealed by this method can differentiate unambiguously between closely related fibers.

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THE APLACOPHORAN FAMILY PROCHAETODERMATIDAE IN THE NORTH AMERICAN BASIN, INCLUDING *CHEVRODERMA* N.G. AND *SPATHODERMA* N.G. (MOLLUSCA; CHAETODERMOMORPHA)*

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ABSTRACT

Six species in three genera of Prochaetodermatidae are described from over 650 stations and 5200 specimens in the Atlantic and north Pacific Oceans from depths between 500 and 7300 m. Included are all species in the North American Basin and all species in *Chevroderma* n.g.

Three principal characters differentiate prochaetodermatid species and genera: spicules, radula, and body shape. Family membership is defined by radula and jaws, spicule morphology determines genus, and species are described by spicules and radula. Mean body shape describes populations of species. Interference colors produced by the aragonite spicules indicate spicule thickness and symmetry. The variation in *Prochaetoderma yongei* n. sp., described in detail, establishes the taxonomic base on which to judge the morphological limits of a prochaetodermatid species.

Spathoderma n.g. and *Chevroderma* n.g. differ from each other and from the genus *Prochaetoderma* in spicule morphology. *P. yongei* and *S. clenchi* n. sp. are widespread northwestern and eastern Atlantic continental slope and abyssal rise species. *C. turnerae* and *C. gauson* n. spp. are abyssal species, the former occurring throughout the Atlantic, the latter only in the northern West European Basin. *C. scalpellum* n. sp. is a slope species of restricted range in the eastern Atlantic. *C. whitlatchi* n. sp. is a wide-ranging abyssal and hadal species of the northern east and mid-Pacific. A wide geographic range is correlated with a vertical depth distribution greater than 1500 m.

All species are patchy in distribution but particular species can be numerically dominant and occur at high densities locally, *e.g.*, up to 400 m^{-2} for *P. yongei* and 178 m^{-2} for *C. whitlatchi.* In the north Atlantic, greatest numerical abundances and lowest diversity of Prochaetodermatidae occur in the North American Basin.

INTRODUCTION

Aplacophoran mollusks belonging to the family Prochaetodermatidae are the most numerous and widespread of the Chaetodermomorpha in the deep sea. They have been taken at all depths in the north and south Atlantic Ocean, Mediterranean Sea, central, north and east Pacific Ocean, and in the western Pacific off southeastern Australia (unpub. data). Particular species are sometimes numerically among the most abundant macrofaunal species in quantitative samples and are thus an important part of the deep-sea fauna (Scheltema, 1981).

Previously the family has consisted of only two named species belonging to the genus *Prochaetoderma: P. raduliferum* (Kowalevsky 1901) from the Mediterranean and *P. californicum* Schwabl 1963 from the troughs off southern California, although other unnamed species have been figured (Treece, 1979, Fig. 5; Scheltema, 1978, Figs.

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1A, 2, 3B; 1981, Figs. 1D, 2E–G, 3D–H, K, 7, 11A–C). Collections taken in the last two decades from both the Atlantic and Pacific Ocean contain several new species and genera belonging to this family.

Six new species in three genera are described here, including all the species that occur in the North American Basin and all those so far collected that belong to the new genus *Chevroderma.* The variation in one species, which has a broad geographic range, is described in detail to establish the taxonomic base on which to judge the morphological limits of a species in the family Prochaetodermatidae.

MATERIALS AND METHODS

Descriptions are based on examination of more than 5200 specimens from the Atlantic Ocean and 68 from the Pacific including the following regions (Tables I, II; Fig. 1): in the Atlantic west of the Mid-Atlantic Ridge—North American Basin, Newfoundland Basin, Brazil Basin, and Argentine Basin; in the Atlantic east of the Mid-Atlantic Ridge—West European Basin, Canary Basin, Cape Verde Basin, Angola Basin, and Namibia Basin; in the eastern Pacific—Panama Basin, Galapagos vents area, off southern California, off Oregon, and Aleutian Trench; and in the mid-Pacific—just north of the Equator.

Samples were taken with a variety of quantitative and nonquantitative gear. The latter included several types of trawls and epibenthic sleds, and the former, box and tube corers manipulated from submersibles and box corers and anchor dredges put overboard (see footnote, Table I).

Most samples were screened using a flotation method (Sanders *et al.,* 1965), fixed in formaldehyde, and preserved in 70–80% alcohol on shipboard. Samples were later sorted into taxa in the laboratory.

Measurements of specimens were made by using dividers or a digitizer on lines drawn length- and crosswise on camera-lucida images at 12× or 25×. Measurements were made to the nearest 0.01 mm in the first two species described, with an accuracy of 0.05 mm; however, it was found that the precision of measurements is not greater than 0.1 mm, and all subsequent measurements are so given.

Treatment of spicules for both light transmission and scanning electron microscopy and of radulae has been previously described (A. Scheltema, 1972, 1976). Permanent preparations of spicules and radulae were made by drying them directly on a slide and mounting with a standard histological plastic medium. Spicules were also examined under cross-polarized light to measure thickness. X-ray diffraction showed them to be formed of aragonite with the long axes of the crystals parallel to the long axis of the spicule, and by comparing the highest interference color with a standard crystallographer's chart, greatest thickness was estimated to the nearest 0.5 μm. Selected isochromes were drawn to show the pattern of thickening and symmetry of the spicules (see Fig. 2C). Length and width of spicules were measured with an ocular micrometer.

Length and width of radula teeth, central plates, and jaws (shown in Fig. 15) were measured with an ocular micrometer.

TYPE MATERIAL

Types are deposited in the National Museum of Natural History, Washington, DC (USNM) and the Muséum National d'Histoire Naturelle, Paris (MNHN). Each holotype is preserved in buffered 80% alcohol after removing a few spicules to a permanent slide. Specimens from the type locality and those used for illustrating spicules

TABLE I

Specimens examined, Atlantic Ocean (Prochaetoderma yongei, Spathoderma clenchi, Chevroderma turnerae, C. gauson, *and* C. scalpellum)

Cruise or dive no.*	Station	Gear**	Date	Depth m	Latitude	Longitude	*P. yongei*	*S. clenchi*	*C. turnerae*	*C. gauson*	*C. scalpellum*
					North American Basin						
					North	West					
ATLANTIS 263	E #3	AD	25/V/61	823	39°50.5′	70°35′	5				
or 264	F #1	AD	24/V/61	1500	39°47′	70°45′	7				
	G #1	AD	24/V/63	2000	39°42′	70°39′	3	1			
	H #2	AD	24/V/61	3752	38°05′	69°36′			1		
ATLANTIS 298	58	AD	7/IX/63	2000	38°34.3′	72°55.0′	3	1			
ATLANTIS II-12	61	AD	20/VIII/64	2000	39°43.3′	70°37.8′	5	2			
	62	ES	21/VIII/64	2496	39°26′	70°33′		5			
	70	ES	23/VIII/64	4680	36°23′	67°58′			6		
	73***	ES	25/VIII/64	1470	39°46.5′	70°43.3′	911***	19			
CHAIN-50	81	ES	2/VII/65	5042	39°41′	66°28′	?1				
	84	ES	4/VIII/65	4749	36°24.4′	67°56′			15		
	85	ES	5/VII/65	3834	37°59.2′	69°26.2′			6		
	87	ES	6/VII/65	1102	39°48.7′	70°40.8′	986				
ATLANTIS II-17	92	ES	13/XII/65	4694	36°20′	67°56′			3		
	95	ES	17/XII/65	3753	38°33′	68°32′			4		
CHAIN-58	103	ES	4/V/66	2022	39°43.6′	70°37.4′	7	18			
	104	AD	4/V/66	2050	39°41.3′	70°35.9′		1			
	105	ES	5/V/66	530	39°56.6′	71°03.6′	1				
ATLANTIS II-24	115	ES	16/VIII/66	2030	39°39.2′	70°24.5′	175	211			
	120	ES	20/VIII/66	5018	34°43.0′	66°32.8′			2		
	121	ES	21/VIII/66	4800	35°50.0′	65°11.0′			6		
	122***	ES	21/VIII/66	4833	35°50.0′	64°57.5′			12***		
	125	ES	23/VIII/66	4825	37°24.0′	65°54.0′			4		
	126	ES	24/VIII/66	3806	39°37.0′	66°47.0′			4		
ATLANTIS II-30	128	ES	16/XII/66	1254	39°46.5′	70°45.2′	45				
	131***	ES	18/XII/66	2178	39°38.5′	70°36.5′	21	71***	?1		
ATLANTIS II-40	175	ES	29/XI/67	4667	36°36′	68°29′			13		
	178	AD	1/XII/67	1839	39°44.8′	70°32.0′	3	5			
CHAIN-88	207	ES	21/II/69	805	39°51.3′	70°54.3′	166				

	209	ES	22/II/69	1501	39°47.6′	70°49.9′	435	6	
	210	ES	22/II/69	2024	39°43.0′	70°46.0′	78	274	
OCEANUS-10	352	SBC	11/VII/76	3600	38°16.5′	69°38.5′			2
	367	SBC	19/VII/76	1764	39°45.5′	70°37.2′	48	11	
	370	SBC	19/VII/76	1815	39°44.9′	70°35.0′	20†	3†	
KNORR-35	340	ES	24/XI/73	3264	38°14.4′	70°20.3′		1	1
	346	ES	3/XII/73	457	39°54.1′	70°10.7′	3		
KNORR-78	Core I	SBC	IV/80	4617	40°21.5′	63°06.2′			1
ALVIN-328; 407-2; 08-18, 19; 436-2; 437-7; 459-6, 11, 12, 15, 16; 460-3, 4, 7, 16; 546-3; 773-A; 774-4; 776-2, 3, 4; 794-2; 834-2	DOS #1	TC, BC	12/VI/72–18/IX/78	1760	39°46′	70°40′	41	6	
ALVIN-603-2, 3		BEB	7/IX/75	2673	39°19′	70°12′		4	
ALVIN-1311 Mud Box 2-80	DOS #2	Exp.	36 mo.	3644	38°18.4′	69°35.6′			2
ALVIN-586-2; 587-1; 589-1, 2; 590-2; 591-1, 2	DWD	BEB	28/VII/75–2/VIII/75	1833–2452	38°45′–38°57′	72°06′–72°34′	14	33	
ALVIN-412-1, 3; 415-1, 2, 3; 454-6	HA	BEB	26/VI/72–13/IX/72	1141–1800	39°20′–39°28′	72°03′–72°13′	9	8	
ALVIN-595-1	HCR	BEB	15/VIII/75	3264	38°46′	71°10′		1	
ALVIN-680-2	RAD	BEB	3/VIII/76	2749	38°30′	72°11′		1	
					Newfoundland Basin				
CHAIN-106	334	ES	30/VIII/72	4400	40°42.6′	46°13.6′			14
					Brazil Basin				
					South	West			
ATLANTIS II-31	155	ES	13/II/67	3730	00°03.0′	27°48.0′			2
	156	ES	14/II/67	3459	00°46.0′	29°28.0′			11

TABLE I (*Continued*)

Cruise or dive no.*	Station	Gear**	Date	Depth m	Latitude	Longitude	*P. yongei*	*S. clenchi*	*C. turnerae*	*C. gauson*	*C. scalpellum*
					Argentine Basin						
					South	West					
ATLANTIS II-60	242	ES	13/III/71	4382	38°16.9′	51°56.1′			5		
	243	ES	14/III/71	3815	37°36.8′	52°23.6′			1		
	245	ES	14/III/71	2707	36°55.7′	53°01.4′			74		
	246	ES	15/III/71	3343	37°15.1′	52°45.0′			2		
	247	ES	17/III/71	5208	43°33.0′	48°58.1′			4		
	259	ES	26/III/71	3305	37°13.3′	52°45.0′			13		
	262	ES	27/III/71	2440	36°05.2′	52°17.9′			13		
					West European Basin						
					North	West					
CHAIN-106	313	ES	17/VIII/72	1500	51°32.2′	12°35.9′	19				
	321	ES	20/VIII/72	2890	50°12.3′	13°35.8′		1			
	323	ES	21/VIII/72	3356	50°08.3′	13°53.7′		10	6		
	326	ES	22/VIII/72	3859	50°04.9′	14°23.8′			11		
	328	ES	23/VIII/72	4426	50°04.7′	15°44.8′			13	8	
	330***	ES	24/VIII/72	4632	50°43.5′	17°51.7′			11	8***	
INCAL	DS-01	ES	15/VII/76	2091	57°59.7′	10°39.8′	2	141			
	DS-02	ES	16/VII/76	2081	57°58.8′	10°48.5′	1	23			
	CP-01	CP	16/VII/76	2040	57°57.7′	10°55.0′		13			
	CP-02	CP	16/VII/76	2091	57°58.4′	10°42.8′		2			
	CP-03	CP	17/VII/76	2466	56°38.0′	11°06.4′		1			
	CP-04	CP	17/VII/76	2483	56°33.2′	11°11.3′		2			
	DS-05	ES	18/VII/76	2503	56°28.1′	11°11.7′		172			
	DS-06	ES	18/VII/76	2494	56°26.6′	11°10.5′		215			
	DS-07	ES	19/VII/76	2884	55°00.7′	12°31.0′		3			
	DS-08	ES	19/VII/76	2891	55°02.0′	12°34.6′		55			
	CP-05	CP	19/VII/76	2884	55°00.4′	12°29.4′		22			
	CP-06	CP	19/VII/76	2888	55°02.3′	12°40.3′		6			
	DS-09	ES	20/VII/76	2897	55°07.7′	12°52.6′		74	1		

	CP-07	CP	20/VII/76	2895	55°03.4′	12°46.2′		5		
	DS-10	ES	27/VII/76	2719	50°12.7′	13°16.6′		2	1	
	CP-09	CP	27/VII/76	2659	50°15.4′	13°15.8′			1	
	OS-01	ES	30/VII/76	2634	50°14.4′	13°10.9′		2	1	
	WS-02	WS	30/VII/76	2498	50°19.3′	12°55.8′		7		
	CP-10	CP	31/VII/76	4823	48°25.5′	15°10.7′			4	
	DS-11	ES	1/VIII/76	4823	48°18.8′	15°11.5′			6	
	CP-11	CP	1/VIII/76	4823	48°20.4′	15°14.6′			3	
	WS-03	WS	1/VIII/76	4829	48°19.2′	15°23.3′				2
	OS-02	ES	2/VIII/76	4829	48°19.2′	15°15.9′			2	1
	CP-12	CP	4/VIII/76	4796	46°00.5′	10°18.3′			1	
	OS-03	ES	4/VIII/76	4798	46°02.5′	10°19.5′			5	
	OS-04	ES	5/VIII/76	4796	46°03.9′	10°12.8′			5	
	WS-05	WS	5/VIII/76	4804	46°03.0′	10°15.7′			1	
	DS-13	ES	6/VIII/76	4822	46°01.9′	10°17.9′			3	
	WS-07	WS	7/VIII/76	4281	47°30.6′	9°37.1′			4	
	WS-08	WS	9/VIII/76	4287	47°30.5′	9°33.7′			1	
	OS-06	ES	9/VIII/76	4316	47°27.3′	9°36.2′			5	
	DS-16	ES	9/VIII/76	4268	47°29.8′	9°33.4′			2	
	OS-07	ES	10/VIII/76	4249	47°31.8′	9°34.3′			26	
	WS-09	WS	10/VIII/76	4277	47°28.8′	9°34.0′			5	
	WS-10	WS	11/VIII/76	4354	47°27.3′	9°39.9′			2	
	OS-08	ES	11/VIII/76	4327	47°29.8′	9°39.2′			4	
NORATLANTE	111-B16	n.d.	n.d.	4512	45°35.2′	3°51.6′			1	
	113-EO13	n.d.	n.d.	4760	45°08′	5°31′			51	
THALASSA-73	Z-400	GBS	22/X/73	1175	47°33.4′	7°19.0′	1			
	Z-445	PBS	27/X/73	1200	48°52.2′	11°07.0′	1			
	Z-459	GBS	29/X/73	1180	48°37.3′	9°53.0′	1			
BIOGAS-III	DS-45	ES	27/VIII/73	4260	47°33.9′	9°38.4′			1	
	DS-47	ES	31/VIII/73	4230	44°26.8′	4°50.7′			1	
	DS-48	ES	31/VIII/73	4203	44°29′	4°54′			4	
	DS-50	ES	1/IX/73	2124	44°08.9′	4°15.9′			1	
BIOGAS-IV	DS-51	ES	18/II/74	2430	44°11.3′	4°15.4′		4		
	DS-52	ES	18/II/74	2006	44°06.3′	4°22.4′	5			
	DS-55	ES	22/II/74	4125	47°34.9′	9°40.9′			1	
	DS-56	ES	23/II/74	4050	47°32.7′	9°28.2′			2	
	DS-60	ES	24/II/74	3742	47°26.8′	9°07.2′			2	

TABLE I (*Continued*)

Cruise or dive no.*	Station	Gear**	Date	Depth m	Latitude	Longitude	*P. yongei*	*S. clenchi*	*C. turnerae*	*C. gauson*	*C. scalpellum*
	DS-61	ES	25/II/74	2250	47°34.7′	8°38.8′		3			
	DS-62	ES	26/II/74	2175	47°32.8′	8°40′		1			
	DS-63	ES	26/II/74	2126	47°32.8′	8°35′		1			
	DS-64	ES	26/II/74	2156	47°29.2′	8°30.7′		2			
	KR-33	KR	24/II/74	2963	47°30.4′	9°06.9′			1		
	KR-35	KR	25/II/74	4140	47°26′	9°08.7′			1		
BIOGAS-V	DS-66	ES	16/VI/74	3480	47°28.2′	9°—′			6		
	DS-67	ES	17/VI/74	4150	47°31′	9°35′			5		
	DS-70	ES	21/VI/74	2150	44°08.8	4°17.4′		2			
BIOGAS-VI	DS-77	ES	24/X/74	4240	47°31.8′	9°34.6′			1		
	DS-78	ES	25/X/74	4706	46°31.2′	10°23.8′			5		
	DS-79	ES	26/X/74	4715	46°30.4′	10°27.1′			6		
	DS-80	ES	27/X/74	4720	46°29.5′	10°29.5′			4		
	DS-82	ES	29/X/74	4462	44°25.4′	4°52.8′			1		
	DS-85	ES	30/X/74	4462	44°23.2′	4°50.8′			6		
	DS-86	ES	31/X/74	1950	44°04.8′	4°18.7′	8				
	DS-87	ES	1/XI/74	1913	44°05.2′	4°19.4′	6	2			
	DS-88	ES	1/XI/74	1894	44°05.2′	4°15.7′	1				
	CP-14	CP	23/X/74	4237	47°32′	9°35.9′			10		
	CP-15	CP	25/X/74	4715	46°32.2′	10°28.5′			4		
	CP-16	CP	25/X/74	4825	46°27.3′	10°25.8′			1		
	CP-17	CP	26/X/74	4706	46°30.8′	10°19.5′			2		
	CP-19	CP	28/X/74	4434	44°24.9′	4°51.3′			3		
	CP-20	CP	29/X/74	4459	44°23.2′	4°51.4′			2		
	CP-22	CP	30/X/74	4475	44°22.9′	4°54.8′			8		
	CP-22A	CP	30/X/74	4475	44°22.9′	4°54.8′			1		
	CP-25A	CP	1/XI/74	1894	44°05′	4°17′	1				
BIOGAS-VIII	KG-142	SBC	18/V/79	2182	47°33.4′	8°39.7′		1			
	KG-144	SBC	to	2225	47°34.2′	8°40.3′		1			
	KG-145	SBC	8/VI/79	2170	47°32.9′	8°39.1′		2			
	KG-157	SBC		2227	47°34.3′	8°39.8′		1			
BIOGAS-IX	KG-173	SBC	16/V/80	2740	47°31.6′	9°04.2′		1			
	KG-174	SBC	17/V/80	2885	47°32.5′	9°05.5′		1			
	KG-178	SBC	18/V/80	2770	47°32.1′	9°05.2′		1	1		
	KG-179	SBC	18/V/80	2730	47°32.2′	9°04.5′		1			

BIOGAS XI	KG-181	SBC	18/V/80	2811	47°31.5′	9°06.4′		2		
	KG-183	SBC	18/V/80	2748	47°31.5′	9°05.1′		1		
	KG-185	SBC	19/V/80	2828	47°32.0′	9°06.3′		1		
	KG-183	SBC	18/V/80	2748	47°31.5′	9°05.1′		1		
	KG-185	SBC	19/V/80	2828	47°32.0′	9°06.3′		1		
	KG-203	SBC	4/X/81	4210	47°34.9′	9°39.8′			2	
	KG-206	SBC	4/X/81	4130	47°35.2′	9°39.5′			1	
	KG-207	SBC	4/X/81	4135	47°35.3′	9°38.5′			6	
	KG-208	SBC	4/X/81	4130	47°35.0′	9°40.0′			4	
	KG-209	SBC	6/X/81	4190	47°34.4′	9°38.9′			3	
	KG-210	SBC	7/X/81	4135	47°35.4′	9°39.2′			2	
	KG-211	SBC	7/X/81	4170	47°34.7′	9°39.1′			4	
	KG-212	SBC	7/X/81	4130	47°34.9′	9°39.3′			1	
	KG-213	SBC	7/X/81	4150	47°34.7′	9°39.0′			2	
	KG-215	SBC	7/X/81	4110	47°34.8′	9°39.9′			3	
	KG-216	SBC	7/X/81	4200	47°35.0′	9°38.5′			1	
	KG-218	SBC	8/X/81	4170	47°34.8′	9°40.3′			1	
MAC 81	No. 14	Exp.	11 mo.	2120	47°33.4′	8°33.7′		1		
SARSIA	65	ES	25/VII/67	1922	46°15′	4°50′	1			
					Canary Basin North	West				
DISCOVERY	6710	ES	19/III/68	2670	27°23.6′	15°39.6′			4	
	6711	ES	19/III/68	2988	27°14.9′	15°36.3′			1	
					Cape Verde Basin North	West				
ATLANTIS II-31	142	ES	5/II/67	1624	10°30.0′	17°51.5′	19			11
	145	ES	6/II/67	2185	10°36.0′	17°49.0′				9
					Angola Basin South	East				
ATLANTIS II-42	195	ES	19/V/68	3797	14°49′	9°56′			5	
	198	ES	21/V/68	4559	10°29′	9°04′			1	
	199	ES	22/V/68	3764	9°47′	10°29′			4	

TABLE I (*Continued*)

Cruise or dive no.*	Station	Gear**	Date	Depth m	Latitude	Longitude	*P. yongei*	*S. clenchi*	*C. turnerae*	*C. gauson*	*C. scalpellum*
	200	ES	22/V/68	2644	9°41′	10°55′		4			1
	201	ES	22/V/68	1964	9°29′	11°34′					22
	202***	ES	23/V/68	1427	9°05′	12°17′	3				59***
WALDA	DS-18	ES	22/VII/71	4079	6°37.5′	8°18.2′			4		
					Namibia Basin						
					South	East					
ATLANTIS II-42	191	ES	17/V/68	1546	23°05′	12°31.5′	66				
	194	ES	17/V/68	2864	22°54′	11°55′		5			
WALDA	DS-04	ES	9/VI/71	4184	21°57.5′	9°22.7′			6		
	DS-08	ES	22/VI/71	3777	21°58.6′	10°16′			3		
TOTAL SPECIMENS							3122	1480	538	19	102

* Woods Hole Oceanographic Institution cruises: ATLANTIS, ATLANTIS II, CHAIN, KNORR, OCEANUS: Woods Hole Oceanographic Institution dives: ALVIN; Centre National de Tri d'Océanographique Biologique (Brest) cruises: INCAL, NORATLANTE, THALASSA, BIOGAS, MAC, WALDA; National Institute of Oceanography (U.K.) cruises: SARSIA, DISCOVERY.

** Quantitative gear, lowered on wire from shipboard: AD—anchor dredge (0.40–1.00 m^2), SBC—spade box corer (0.25 m^2), KR—Reineck corer (600 cm^2); manipulated from submersible: BEB—Birge-Ekman box corer (25 m^{-2}, 44.5 m^{-2}), TC—tube corer (35 cm^2), BC—box corer (225 cm^2). Nonquantitative samplers: ES—epibenthic sled trawl, CP—perch trawl, WS—Wormley trawl, GBS, PBS—large, small Boillot sled trawls. Experimental boxes: Exp.

*** Type locality.

† Only inner 90 cm^2 of box core sorted.

TABLE II

Material examined, Pacific Ocean (Chevroderma whitlatchi)

Cruise or dive no.*	Station or core	Gear**	Date	Depth m	Latitude north	Longitude west	No.
Aleutian Trench							
SEVENTOW Leg 7	H-39	SBC	20/VII/70	7298	50°58.0′	171°37.5′	31
Off Oregon							
Oregon State U.	AD-136	AD	9/II/65	2800	44°50.9′	127°34.1′	1
Off Baja California							
MELVILLE 70-III-I	n.d.	n.d.	21/III/70	3950	31°47.0′	120°14.8′	1
Panama Basin							
ALVIN 1132, 1230–32, 1234–39	***	BC	9/IX/81–14/VI/82	3912	5°20.7′	81°56.2′	31***
Near Galapagos Vents							
GILLISS-301	Core 14	SBC	18/II/79	2730	00°35.0′	86°05.7′	1
Mid-Pacific							
DOMES Proj.	48–22	SBC	n.d.	5117	8°16.0′	151°11.3′	2
	53–45	SBC	n.d.	4985	11°43.9′	138°22.2′	1

* Scripps Institution of Oceanography cruises: SEVENTOW, MELVILLE 70; Woods Hole Oceanographic Institution dives: ALVIN; Galapagos Biology Expedition cruise: GILLISS; U.S. National Oceanographic and Atmospheric Administration project: DOMES.

** See footnote, Table I.

*** Type locality.

and radulae are designated as paratypes; for most illustrated paratypes there are permanent slides of spicules and radulae.

TAXONOMIC CHARACTERS

The taxonomy of the Aplacophora has in the past relied primarily on interpretation of histological sections, and most species descriptions have been based on one or a few specimens. The results have not made for easily accessible or recognizable characters for purposes of identification. Radulae and spicules have seldom been adequately figured, and no studies exist that rigorously describe intraspecific variation within a species.

The acquisition of large deep-sea benthic samples over wide geographic areas since the early 1960s has made numerous specimens available for studying aplacophoran species both between and within populations. The insights gained from these large and numerous samples have shown that for the Prochaetodermatidae, hard parts and

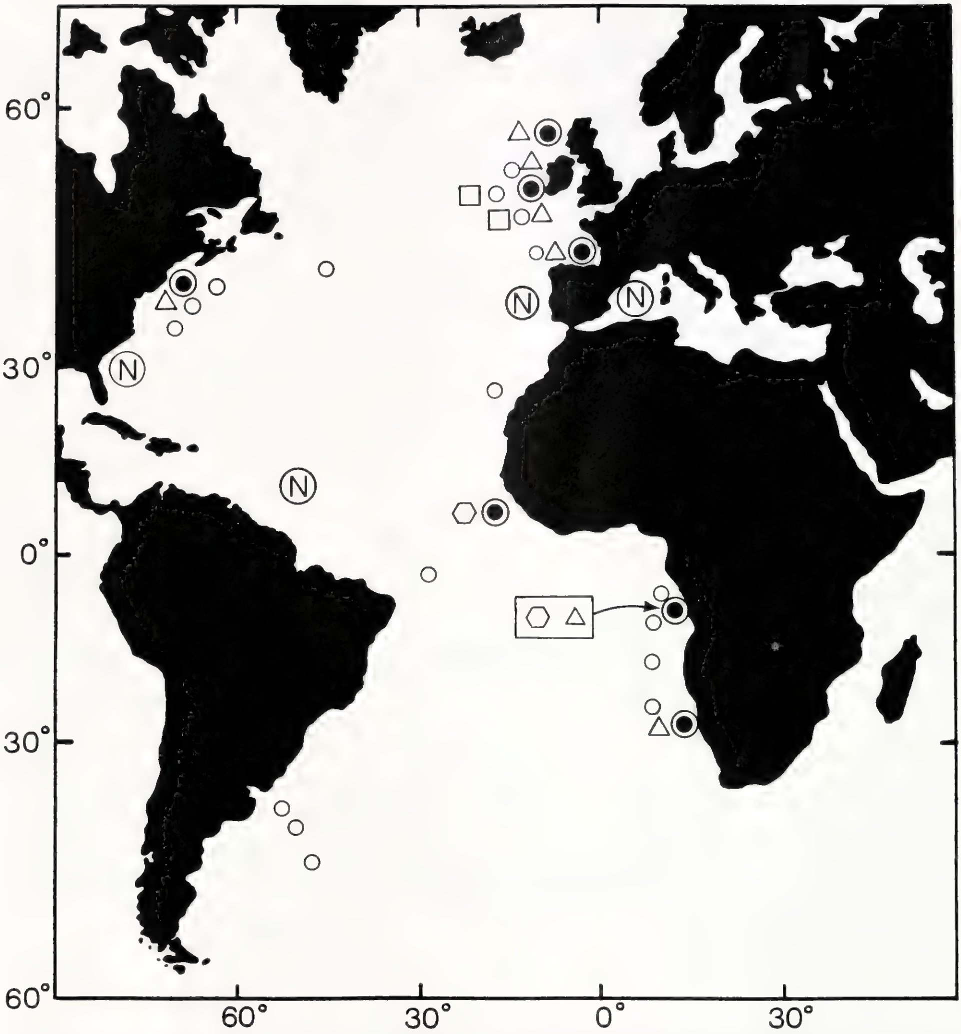

FIGURE 1. Distribution of *Prochaetoderma yongei* (solid, ringed circle), *Spathoderma clenchi* (triangle), *Chevroderma turnerae* (open circle), *C. gauson* (square), and *C. scalpellum* (hexagon). Data generalized from about 165 stations listed in Table I. Circled N, area sampled in which none of the five species occurred.

external morphologies characterize species, and that the morphology of spicules determines affinities and defines genera in this family.

No internal soft anatomy has been used here to describe species because of the great histologic similarity among species and because the size and arrangement of internal organs is reflected in the body shape.

Body shape

The body of the Prochaetodermatidae is cylindrical and divided into three regions: anterium, trunk, and posterium (Fig. 2A). The *anterium* bears the oral shield and few

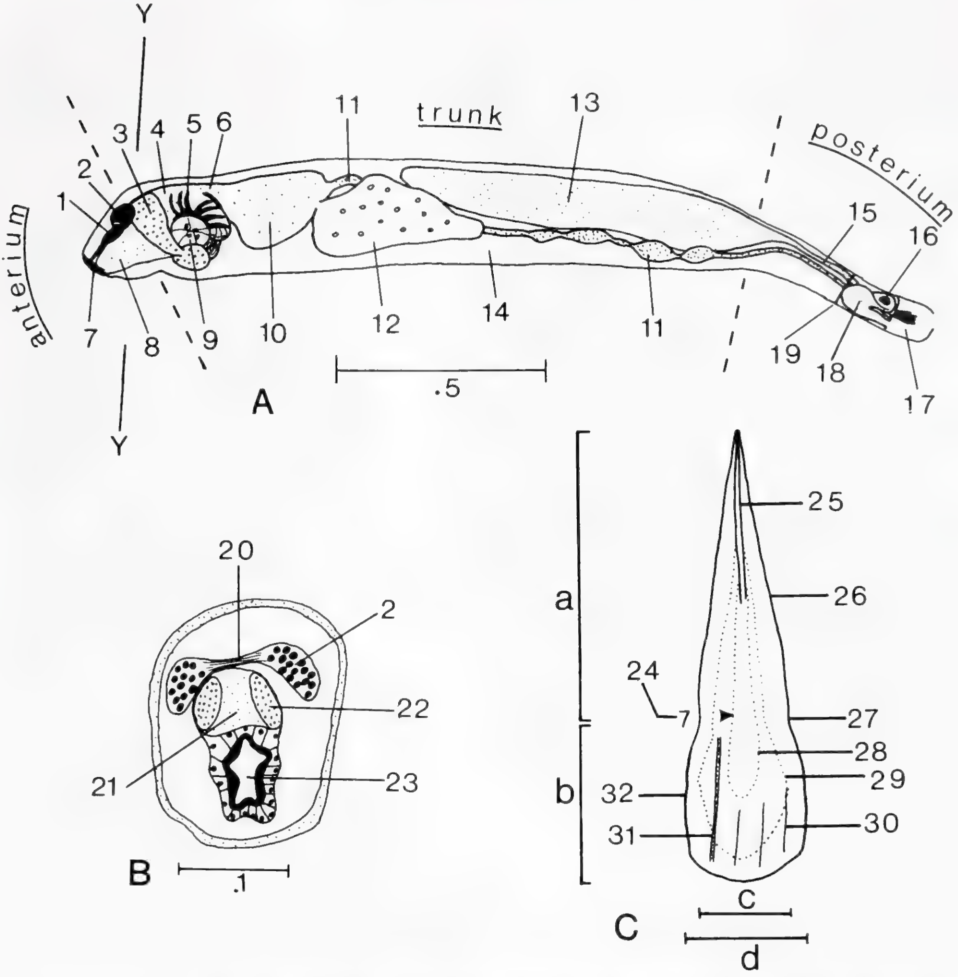

FIGURE 2. A. Genus *Prochaetoderma,* generalized anatomy, semi-diagrammatic. Dashed lines indicate boundaries between body regions. B. Cross-section through Y-Y in diagram A showing distal ends of jaws and separated lobes of cerebral ganglia joined by thick commissure. C. Morphological characters of a spicule. 1 precerebral ganglion, 2 cerebral ganglion, 3 jaw, 4 buccal cavity, 5 radula, 6 esophagus, 7 oral shield, 8 oral cavity, 9 chondroid-like bolster, 10 stomach, 11 intestine, 12 digestive gland, 13 gonad, 14 hemocoele, 15 gonopericardial duct, 16 heart within pericardium, 17 cloaca or mantle cavity with gill, 18 coelomoduct, 19 vertical posterior septum, 20 cerebral commissure, 21 sheet of cuticle joining distal ends of jaw, 22 distal end of jaw, 23 cuticle-lined oral cavity, 24 greatest spicule thickness in micrometers indicated by arrowhead and numeral, 25 keel or heavy ridge, 26 blade edge, 27 waist, 28 and 29 isochromes, 30 fine ridge, 31 groove, 32 base edge, *a* blade length, *b* base length, *c* maximum blade width, *d* maximum base width.

spicules and is often inflated; it may be retracted and introverted in preserved specimens. The *trunk* is set off from the anterium by a much greater density of spicules. The *posterium* is narrower than the trunk and often elongate and tail-like; the exact demarcation between trunk and posterium is sometimes difficult to establish.

Measurements of the trunk and posterium allow quantification of the variation in body shape among populations of a single species, as well as differentiating body shape among species.

Oral shield

The paired oral shield varies in size among species of Prochaetodermatidae (Fig. 3); size is also related to specimen size within a species and thus must be used cautiously as a taxonomic character. The relative sizes among species can be compared quanti-

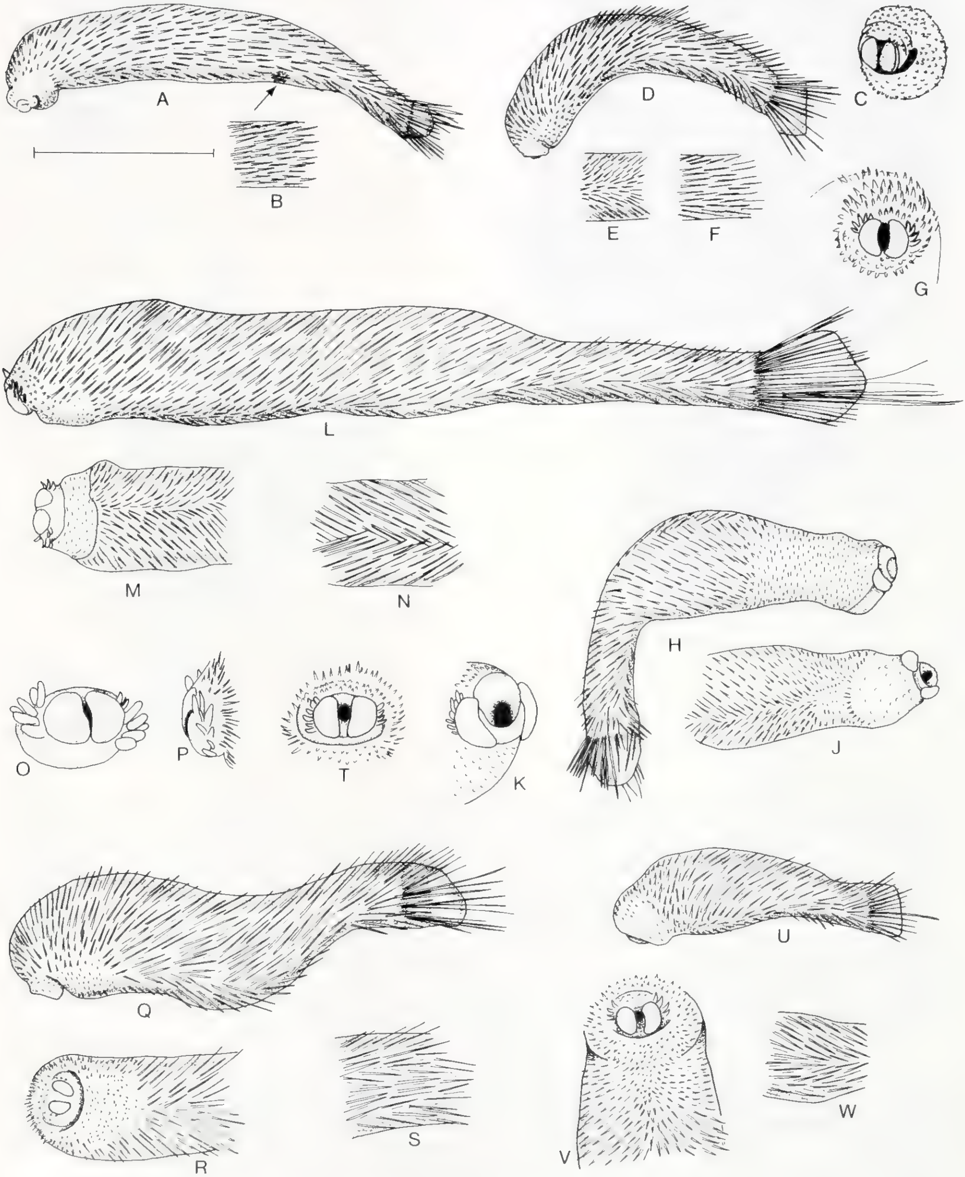

FIGURE 3. Holotypes. A–C. *Prochaetoderma yongei* n. sp. D–G. *Spathoderma clenchi* n.g. n. sp. H–K. *Chevroderma whitlatchi* n.g. n. sp. L–P. *Chevroderma turnerae* n. sp., genus type. Q–T. *Chevroderma gauson,* n. sp. U–W. *Chevroderma scalpellum* n. sp. Distribution of spicules, mid-dorsal view: B, F, N, S, W; mid-ventral view: E, M, J, R, V. Scale line equals 0.5 mm for oral shield illustrations C, G, K, O, P, T, and V; for all remaining illustrations it equals 1.0 mm.

tatively by using the following index: (length × width of oral shield) ÷ (trunk diameter) × 100. This index is given for holotypes.

There may be relatively large, possibly tactile oral-shield spicules just lateral to the oral shield (Figs. 3G, K, O, P); they are lacking or indistinct in some species (Fig. 3C).

Cloaca, or mantle cavity

The posterior end of the body is rounded or truncated in lateral view, the shape depending on the cloacal wall musculature which closes off the mantle cavity (Fig. 3).

Spicules

Attitude. According to species, spicules are carried flat against the body or bent outwards from it. They may be arranged with the long axis parallel to the body axis (Fig. 3A) or obliquely with the proximal end ventral to the distal end (Fig. 3L). A species may be translucent or opaque according to spicule thickness and attitude. The morphology of the spicules and body wall musculature determine the attitude of spicules relative to the body wall.

Morphology. The morphology of individual spicules is the taxonomic character most useful for distinguishing species and for determining relationships among species. A spicule has a *base* (Fig. 2C), which lies within the cuticle and is anterior in position to the *blade,* which is external to the cuticle; the base is usually set off from the blade by a *waist.* The blade may have a *keel* on the frontal surface; *ridges* or *grooves* may be present on either the blade or base. Spicules may be symmetrical or asymmetrical. Symmetry is revealed by interference colors seen under cross-polarized light. Spicules may lie flat in one plane, or the blade may be rotated about the long axis to a plane different from that of the base or it may be bent at the waist towards the body or away from it.

Spicules vary in their morphology both along the body from anterior to posterior and from the ventral to the lateral and dorsal sides; thus they are here described from particular regions of the body, which are indicated on a drawing of the specimen from which the spicules were taken (Figs. 6–13). However, dorsal spicules at the junction of the trunk and posterium are usually adequate for species determination and their morphology alone is given under the diagnosis for each species.

Radula and jaws

The jaws and radula of the family Prochaetodermatidae have been described by Kowalevsky (1901) and Scheltema (1981). There is great morphological similarity among species in these structures, the greatest differences lying in size of jaws and teeth and in shape and length of the central plate (Figs. 14, 15). No statistically significant correlations were found in mature specimens of two species (a) between specimen length and either tooth or jaw length, or (b) between tooth length and jaw length. The ranges in ratios of tooth length to jaw length are similar in all six species described here.

The jaws can usually be seen *in situ* within a specimen viewed with transmitted light and thus family membership determined without dissection.

SYSTEMATIC ACCOUNT

Family Prochaetodermatidae Salvini-Plawen 1969

The Prochaetodermatidae are Chaetodermomorpha (= Caudofoveata), or burrowing, footless solenogasters, uniquely characterized by the presence of a pair of large, cuticular jaws and the morphology of a small distichous radula. The cuticle of the jaws is discrete from the buccal cavity cuticle (Scheltema, 1981); it is bound by basement membrane and not produced by preradular, lateral pouches as stated in Boss (1982). The radula is formed of 8 to 12 rows of paired teeth on an undivided radular membrane; between each pair is a central plate. A lateral, tooth-like projection of the radular membrane lies alongside each tooth (Fig. 15 upper left tooth; Scheltema, 1981, Figs. 7D, 11A). Each tooth has a lateral membranous "wing" and a membranous median extension, or brush, bearing many serrations (Figs. 4E, 15). The distal teeth are worn (Scheltema, 1981, Fig. 11B). The odontophore bolsters are chondroid-like (Fig. 2A; Scheltema, 1978, Fig. 3B).

All prochaetodermatid species are small, usually less than 5 mm in body length, and thicker anteriorly than posteriorly. Although the body is cylindrical, the dorsal and ventral surfaces are distinct: the spicules diverge away from the ventral midline and the body of contracted specimens is usually flexed into an arch, the dorsal side uppermost. The oral shield is divided into two lateral parts with or without lateral spicules. The epidermal spicules are solid, with ornamentation simple or lacking; the base is flat in cross-section and embedded in the epidermal cuticle; the blade is flat, oval, or triangular to round in cross-section and extends beyond the cuticle. Paired groups of long, lateroventral spines trail posteriorly beyond the cloaca (Figs. 3L, U) but are often broken off in preserved specimens.

Three distinct body regions reflect internal anatomy (Fig. 2A): (1) the anterium is a hemocoelic space surrounding an expansible oral cavity into which can be protruded the jaws and buccal mass (Fig. 10, specimen); (2) a broad trunk, with weak body-wall musculature, contains the paired lobes of the cerebral ganglion with their thick commissure (Fig. 2B), buccal mass, stomach, digestive gland, anterior intestine, and gonad; and (3) a narrow posterium, with strong longitudinal muscles, contains the posterior intestine, unpaired gonoduct (paired in other chaetodermatids), heart, pericardium, paired coelomoducts, and, posteriorly, a small cloaca with a pair of gills, each formed of two lamellae (Salvini-Plawen, 1969, Fig. 12). The stomach is unique among the Chaetodermomorpha in lacking a dorsal ciliated typhlosole, and it has no locally thickened cuticle; the digestive gland is uniquely without a dorsal band of granular cells (Scheltema, 1981). The members of the family are dioecious; in translucent species, sex can often be determined without dissection.

Except in the Scandinavian fjords, Arctic, and Antarctic where they have not been reported, the Prochaetodermatidae are ubiquitous in soft oceanic sediments from 50 to over 7000 m.

Genus *Prochaetoderma* Thiele

Prochaetoderma Thiele 1902, *Zeit. Wiss. Zool.* **72II:** 275.

With characters of the family. Spicules flat; base shorter than blade, blade broad and triangular with median keel and sharp distal point.

Distribution: Atlantic Ocean and Mediterranean Sea, 50–2000 m.

Type species: *Chaetoderma radulifera* Kowalevsky 1901, by monotypy; Sea of Marmara.

Prochaetoderma raduliferum (Kowalevsky)

Chaetoderma radulifera Kowalevsky 1901, *Arch. Zool. Exp.*, ser. 3, **9:** 264–274, Figs. 1–20. Sea of Marmara, Isle des Princes, 35–40 fms. [Type specimen unknown; type figure, here designated, P1. 10, Fig. 3; redrawn herein, Fig. 6].

Prochaetoderma raduliferum. Salvini-Plawen, 1972, pp. 37–39, Figs. 10–12 in *Fifth European Marine Biology Symposium,* B. Battaglia, ed. Piccin Editore, Padova.

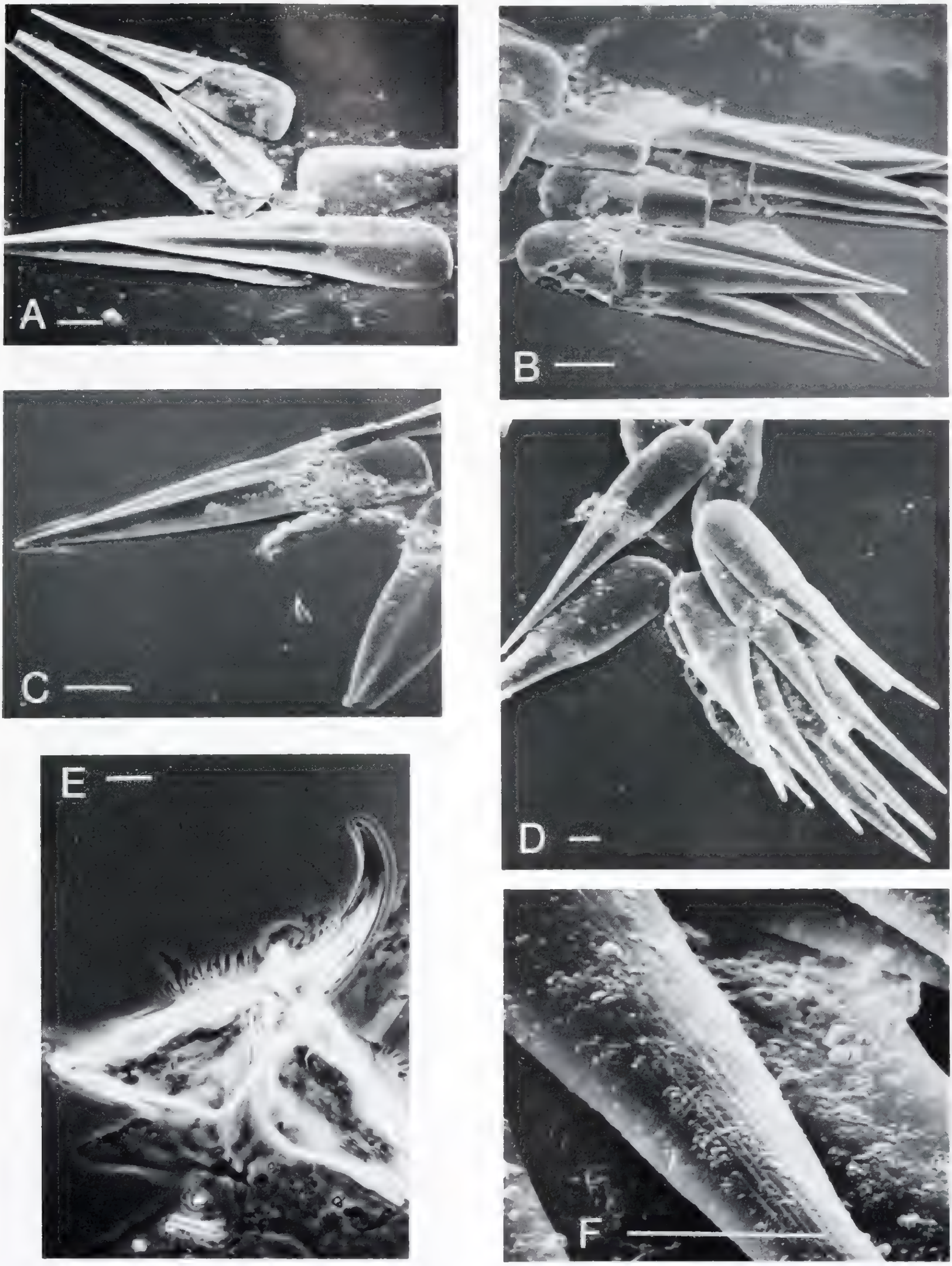

FIGURE 4. Spicules of *Prochaetoderma yongei* n. sp. (A–C) and *Spathoderma clenchi* n.g., n. sp. (D, F); radula tooth (E) of an unnamed species of Prochaetodermatidae. A. *P. yongei* holotype, 1470 m, North American Basin (*cf.* Fig. 6); B. *P. yongei,* 805 m, North American Basin (CH-88 Sta. 207) (*cf.* Fig. 7); C. *P. yongei,* 1546 m, Namibia Basin (AII-42 Sta. 191) (*cf.* Fig. 8); D. *S. clenchi* holotype, 2178 m, North American Basin (*cf.* Fig. 9); F. same as D, detail of blade. Scale lines equal 20 μm.

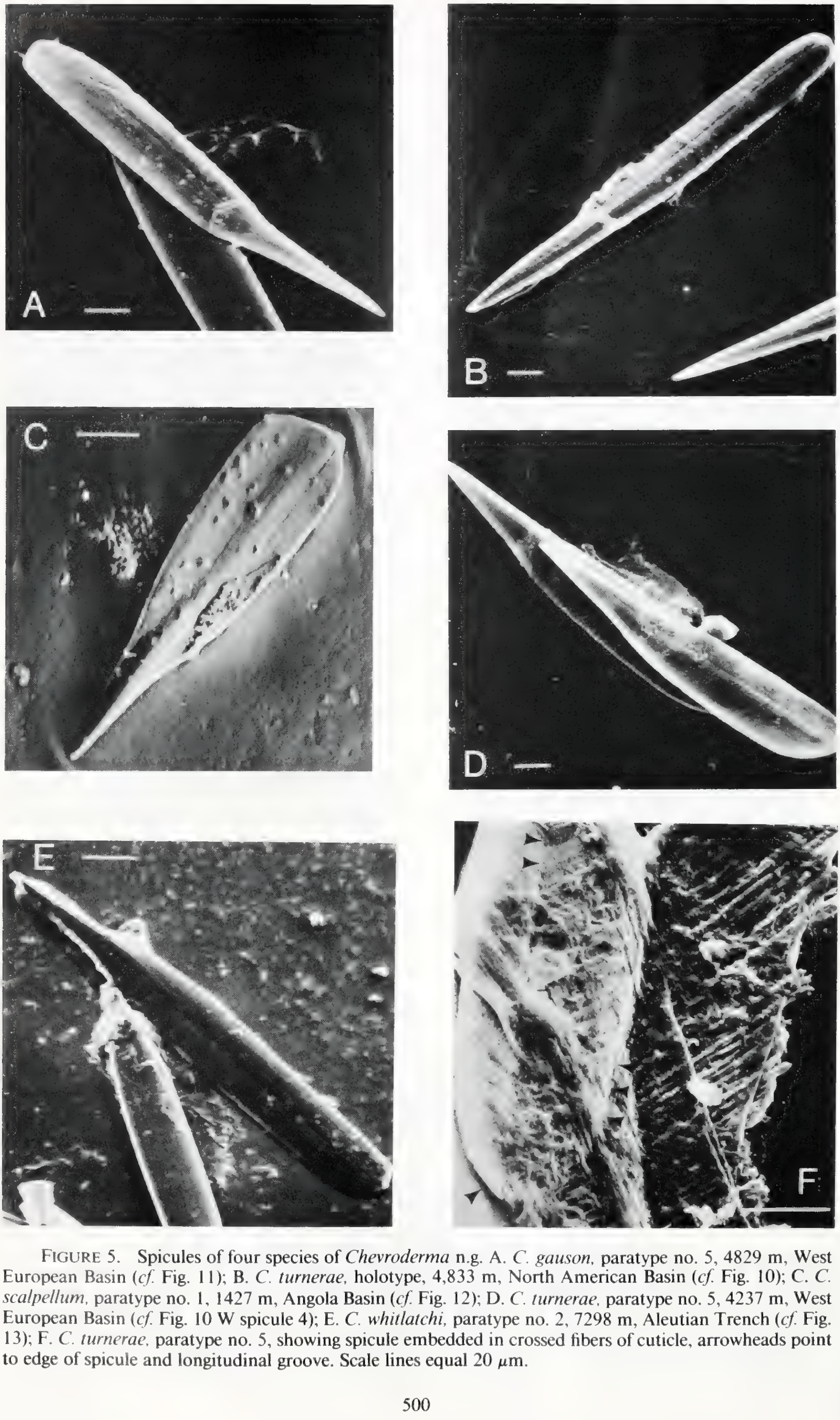

FIGURE 5. Spicules of four species of *Chevroderma* n.g. A. *C. gauson,* paratype no. 5, 4829 m, West European Basin (*cf.* Fig. 11); B. *C. turnerae,* holotype, 4,833 m, North American Basin (*cf.* Fig. 10); C. *C. scalpellum,* paratype no. 1, 1427 m, Angola Basin (*cf.* Fig. 12); D. *C. turnerae,* paratype no. 5, 4237 m, West European Basin (*cf.* Fig. 10 W spicule 4); E. *C. whitlatchi,* paratype no. 2, 7298 m, Aleutian Trench (*cf.* Fig. 13); F. *C. turnerae,* paratype no. 5, showing spicule embedded in crossed fibers of cuticle, arrowheads point to edge of spicule and longitudinal groove. Scale lines equal 20 μm.

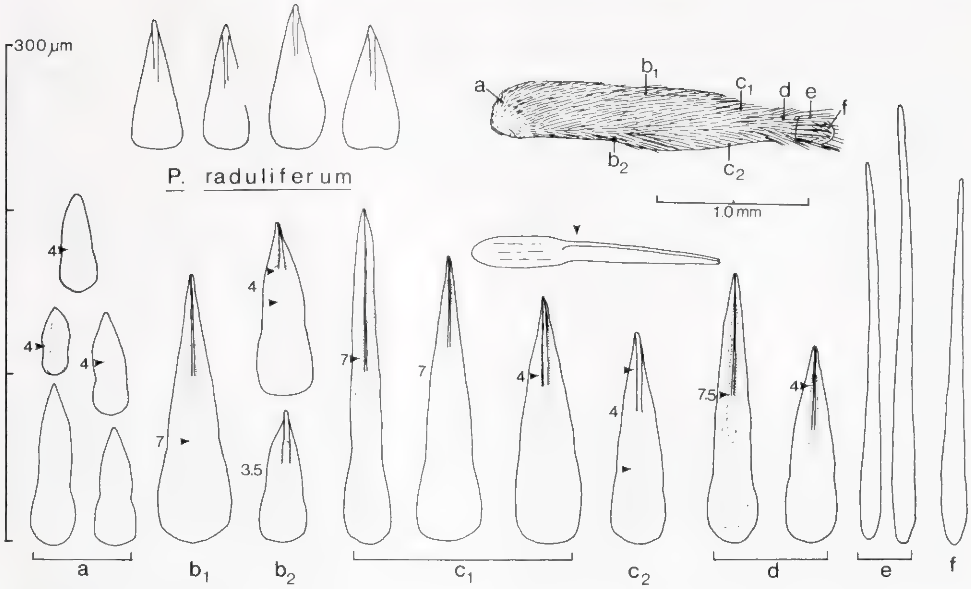

FIGURE 6. *Upper left:* spicules of *Prochaetoderma raduliferum* (Kowalevsky 1901) redrawn from type figure (pl. 10, Fig. 3); scale of original drawings not certain, scale used here from Salvini-Plawen (1972, Fig. 12 d, f). *Lower rows:* spicules of *Prochaetoderma yongei* n. sp. paratype no. 1, 1470 m, North American Basin (type locality). Spicules *a–f* from body regions indicated on figured specimen, above right; spicule below specimen, lateral view of a trunk spicule showing blade slightly offset outwards from base (arrowhead).

Prochaetoderma raduliferum. Salvini-Plawen, 1977, *Vie Milieu* **27:** 56–63, Figs. 3–5; *Bull. Mus. Nat. Hist. Nat., Paris* (ser. 3), **447** (Zool. 310): 417, Table I.
Distribution: Sea of Marmara; eastern and western Mediterranean; 54–2415 m.

Prochaetoderma yongei n. sp.

Figs. 1, 3A–C, 4A–C, 6, 7, 8, 14 y^1–y^3; Tables I, III, IV, V, VII

Prochaetoderma sp. Grassle, 1977, *Nature* **265:** 618–619, Table 2.
Prochaetoderma sp. Scheltema, 1978, *Malacologia* **17,** Figs. 1A, 3B, 5C.
Prochaetoderma sp. *y.* Scheltema, 1981, *Malacologia* 20, Figs. 2E–G, 3D–H, K, 7D–F, 11A–C (*non* 7A–C, caption in error).
Prochaetoderma sp. B. Scheltema, 1985, *in* L. Laubier and C. Monniot, eds., *Peuplements Profonds du Golfe de Gascogne: Campagnes BIOGAS,* IFREMER, Brest, pp. 391–396, Tables 1, 2.

Diagnosis: Translucent, slender; spicules flat-lying, parallel to long axis of body; less than 3 mm long, posterium ¼ total length; posteroventral thickened cuticular patch present; oral shield small, oral shield spicules not distinct; spicules straight, sharply keeled, indented at waist, blade with convex sides, base flared proximally, greatest length 252 µm; greatest jaw length 365 µm; radula tooth length up to 90 µm; central radula plate short and relatively broad with ends directed posteriorly, greatest length 26 µm.

This species is named in honor of Sir Maurice Yonge, whose book *The Seashore* early led me to the study of marine animals.

Holotype: North American Basin, 39°46.5'N, 70°43.3'W, 1470–1330 m (ATLANTIS II-12, Sta. 73, 25/VIII/64). USNM No. 850201.

Illustrated paratypes:

Nos. 1, 4: Type locality, USNM Nos. 850202 (No. 1), 850208 (No. 4).
No. 2: Namibia Basin, 23°05'S, 12°31.5'E, 1546–1559 m (ATLANTIS II-42 Sta. 191, 17/V/68). USNM No. 850204.
No. 3: North American Basin, 39°51.3'N, 70°54.3'W, 805–811 m (CHAIN-88 Sta. 207, 21/II/69). USNM No. 850206.

Description

External morphology. Prochaetoderma yongei is a small, slender, translucent species with flat-lying spicules oriented anterior-posterior except where they diverge along the ventral midline (Fig. 3A, B). Oral shield spicules are indistinct. The total body length averages 1.5 to 2.1 mm in seven populations; the greatest length is 2.8 mm. Trunk diameter averages 0.3 to 0.4 mm, with greatest diameter 0.6 mm. The posterium is about ¼ total length; it averages 0.4 to 0.5 mm in length and 0.2 mm in diameter and ranges up to 1.0 mm by 0.3 mm. The mean index of posterium length to trunk length averages 0.31 to 0.35 in seven populations, but the range is great, from 0.18 to 0.68. An opaque, thickened patch of cuticle at the ventral junction of the trunk and posterium is characteristic of the species (Fig. 3A, arrow). The oral shield is small (Fig. 3C); the cloaca is rounded.

Holotype: Male; total length 2.6 mm; trunk 2.0 by 0.4 mm; posterium 0.6 by 0.2 mm; index of posterium to trunk 0.30. Oral shield 0.05 by 0.09 mm; index of oral shield to trunk diameter 1.12.

Spicules. A median keel runs the length of the blade; it is sharpest and most distinct distally (Figs. 4A–C, 6, 7, 8) and is lacking in anteriormost and posteriormost spicules from regions *a, e,* and *f.* Other ornamentation on the blade is lacking except in region b_1 from specimens in the Namibia Basin, which have short ridges parallel to the keel (Fig. 8). The spicule base is flared proximally with convex sides and sometimes faint ridges; occasionally the base has straight sides which are either flared or parallel. The proximal end is usually rounded, but may be straight or broadly triangular. The blade usually has convex sides proximally; it is distinctly set off from the base by an indentation at the waist. Some blades have straight sides, particularly in juveniles (Fig. 7D). Distally the sides of the blade straighten or become slightly concave before tapering to a sharp point. The isochromes are symmetrical. Spicules from the anterior end of the body are thickest at the proximal part of the blade just above the waist (region b_1); further posteriorly, they are thickest more distally (regions *c* and *d*). In lateral view the base and blade are nearly straight, with the blade somewhat offset outwards from the base and slightly bent towards the body. Spicules from the ventral side of the body are thinner and shorter than those from the lateral and dorsal sides, and often the keel extends like a needle beyond the sides of the blade (regions b_2, c_2). Very short, very thin (2 μm) triangular spicules occur sparsely.

Ranges in dimensions of spicules from region *c* for eight populations in five ocean basins are shown in Table III.

The spicules of *P. yongei* differ from those figured by Kowalevsky (1901) for *P. raduliferum* in having a longer blade and a distinct waist (Fig. 6). The sides of the

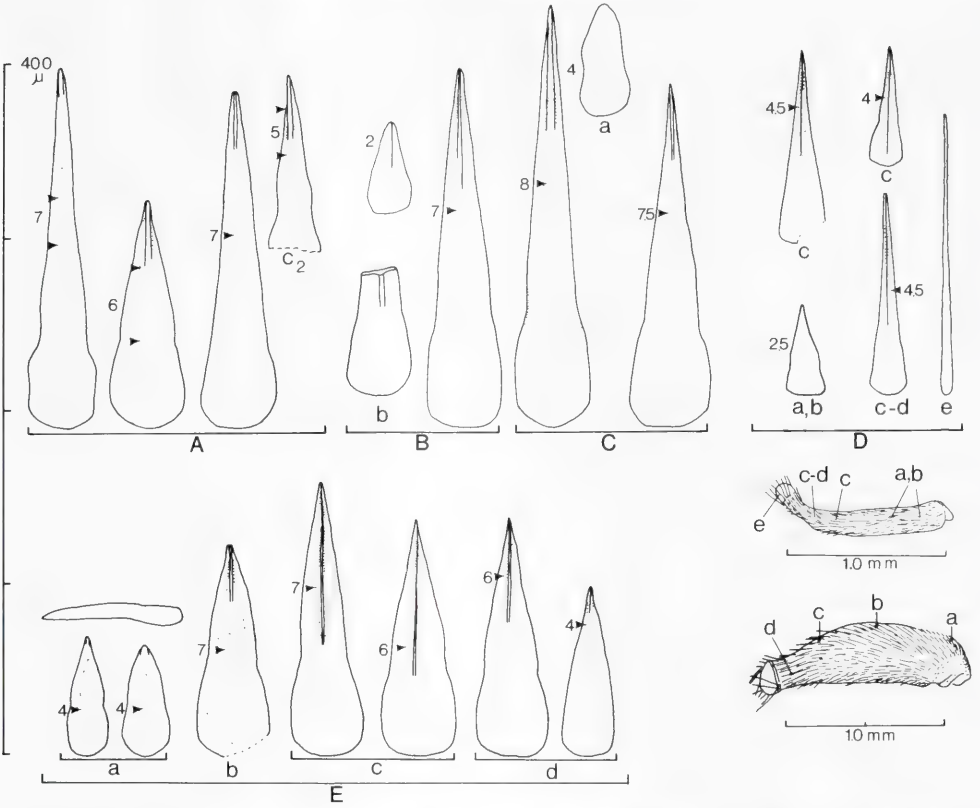

FIGURE 7. Variation in spicule morphology of *Prochaetoderma yongei* n. sp. from the North American Basin. A–C. spicules from junction of trunk and posterium, unless indicated otherwise; A. from single specimen from 805 m (CH-88 Sta. 207); B. from 3 specimens from 1470 m (type locality); C. from single specimen from 530 m (CH-58 Sta. 105); D. spicules from paratype no. 4, figured just below, immature specimen (type locality); E. spicules from paratype no. 3 figured at lower right, a short, stubby specimen from 805 m (CH-88 Sta. 207).

blade are straight in *P. raduliferum* and convex in *P. yongei;* the base in *P. raduliferum* is round, and in *P. yongei* it is flared proximally.

Radula and jaws. The jaws and teeth are typical for the family and small (Fig. 14 y^1–y^3). Tooth length ranges up to 90 μm; jaw length and width range up to 365 and 128 μm, respectively. The entire radula has 8 to 11 rows of teeth. The central radula plate is short, up to 26 μm, and broad, up to 8 μm; the ends are directed posteriorly.

Morphological variation

Several populations of *Prochaetoderma yongei,* identified by similarity of spicule and radula morphology, were examined to determine the variability that occurs within this species and could therefore be expected in other species of the family Prochaetodermatidae.

Spicules. Several spicules from the dorsal junction of trunk and posterium were drawn and compared for 24 specimens from four stations along the Gay Head-Bermuda transect in the North American Basin and from five stations between the northern

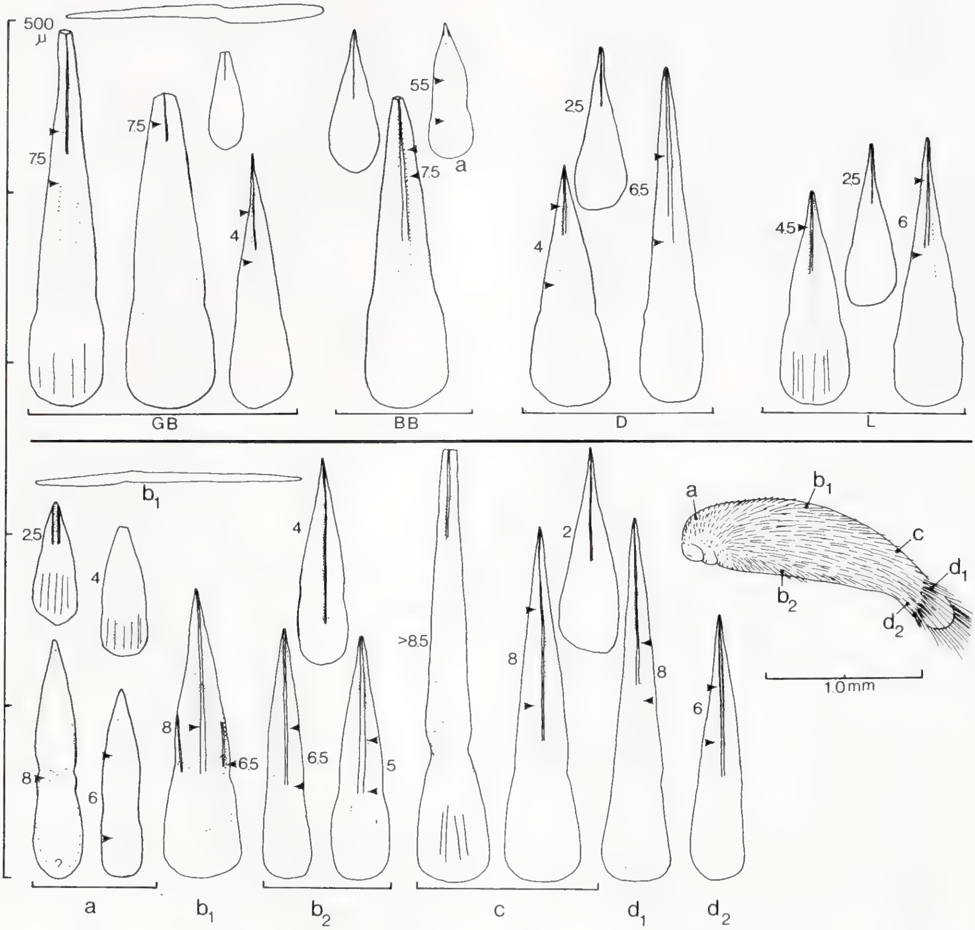

FIGURE 8. Variation in spicule morphology of *Prochaetoderma yongei* n. sp. from the eastern Atlantic. *Upper row:* spicules from junction of trunk and posterium, unless otherwise indicated. GB. off Great Britain (West European Basin), 2091 m (INCAL DS-01); BB. Bay of Biscay (West European Basin), 1922 m (SARSIA 65); D. Cape Verde Basin, 1624 m (AII-31 Sta. 142); L. Angola Basin, 1427 m (AII-42 Sta. 202). *Lower row:* spicules from paratype no. 2, 1546 m, Namibia Basin. Upper and middle left, lateral views showing offset blade.

West European Basin and Namibia Basin in the eastern Atlantic; spicules were measured from fifteen of these specimens (Table III) and scanning electron photomicrographs were made of spicules from three (Fig. 4A–C). Variation in shape is as great among spicules from a single specimen (Fig. 7A) as among spicules from far-distant populations (*e.g.,* Namibia Basin at 1546 m, Fig. 8, lower row, and North American Basin at 530 m, Fig. 7C). Greatest blade length decreases with depth in the western Atlantic and from north to south in the eastern Atlantic between the West European Basin and Angola Basin; in the Namibia Basin, blade length again increases (Table III, Fig. 8).

Spicule length is not correlated with body length, except that very small juveniles have relatively small spicules (Fig. 7D). In a test of significance, the longest spicule

TABLE III

Range in spicule dimensions from body region c, *in* Prochaetoderma yongei *and* Spathoderma clenchi *(μm) (number of specimens in parentheses)*

	Total length	Blade length	Base length	Blade width	Base width
		P. yongei			
No. American Basin					
AII-12 Sta. 73 (6) (1400 m)	101–207	60–148	38–70	20–34	27–47
CH-88 Sta. 207 (3) (800 m)	128–202	81–158	40–61	22–32	34–45
CH-58 Sta. 105 (1) (500 m)	202–241	148–178	54–63	27–32	40–50
West European Basin					
INCAL DS-01 (1)	151–225	99–164	45–63	22–43	34–52
SARSIA 65 (1)	184–220	112–153	68–72	32–36	47–50
Cape Verde Basin					
AII-31 Sta. 142 (1)	144–198	88–142	56	25–29	38–47
Angola Basin					
AII-42 Sta. 202 (1)	126–162	74–108	50–52	29–32	40–43
Namibia Basin					
AII-42 Sta. 191 (1)	178–252	122–173	50–79	29–34	40–45
		S. clenchi			
No. American Basin					
AII-30 Sta. 131 (1) (2178 m)	101–202	43–119	68–90	22	27–43
AII-12 Sta. 73 (4) (1400 m)	128–232	56–155	72–90	16–25	34–50
West European Basin					
INCAL DS-01 (1)	122–232	54–146	68–86	11–22	32–45
Namibia Basin					
AII-42 Sta. 194 (1)	142–227	74–130	56–97	22	25–43

from a specimen was not correlated with specimen length in nine North American Basin specimens from 1470 m (AII-12 Sta. 73, $r = .25$) and in 6 specimens from 805 m (CH-88 Sta. 207, $r = .36$). In the specimens from the 805 m station mean body length was 1.6 mm and greatest length of spicules was 202 μm, whereas the mean body length of the specimens from 1470 m was significantly greater, 2.1 mm ($P < .02$), but greatest spicule length was about the same, 207 μm. On the other hand, in the eastern Atlantic, body length of a specimen from 2091 m off Scotland (INCAL

DS-01) was 1.6 mm and greatest spicule length 225 μm, whereas a specimen of the same body length from 1427 m in the Angola Basin (AII-42 Sta. 202) had spicules with greatest length of only 162 μm (Fig. 8GB and L).

It is concluded that spicule shape and ornamentation, but not size, are of taxonomic significance in defining a widely distributed species of Prochaetodermatidae.

Radula. Jaws and radulae of 20 specimens ranging in length from 1.1 to 2.5 mm were examined, 19 from the North American Basin and 1 from the Namibia Basin. Of the former, 6 were from short, stubby specimens at 805 m and 13 from long, slender specimens at 1470 m (*cf.*, specimens in Figs. 6 and 7). For the North American Basin, greatest jaw length at 805 and 1470 m depths was 365 and 360 μm, respectively; greatest tooth length, 90 and 85 μm; and greatest length of the central plate, 25 and 26 μm. In the specimen from the Namibia Basin, jaw length was 336 μm, tooth length 83 μm, and length of central plate 25 μm. Morphology of the central plate was similar in all three locations (Fig. 14, y^1–y^3).

The differences in size among radulae and jaws and in morphology of the central plate seem too slight in the three locations to indicate species differences.

Body shape. Body shape is difficult to quantify in the highly contractible and extensible species of Prochaetodermatidae. The only measurements that are correlated to each other in all populations measured are posterium length to trunk length ($P < .02$ to $<.001$); other measurements such as trunk diameter to trunk length are not correlated at all, or are correlated in only some populations. Yet specimens with similar spicule morphology from different populations may appear to have a distinctive body shape, *e.g.*, short and stubby from 805 m in the North American Basin or long and slender 600 meters further downslope (Figs. 6, 7). Differences between mean values for the characters measured are statistically significant for some characters between some populations, but there is no consistent or clinal pattern (Table IV).

Therefore, to compare body shapes, an overall coefficient of similarity was determined for each of seven populations of *Prochaetoderma yongei,* identified as such by spicule morphology, and for comparison, for one population of *Spathoderma clenchi,* n. sp. (*q.v.*) which is very similar in shape but not in spicule morphology. The mean character difference (M.C.D.) was computed from five characters: trunk length, trunk diameter, posterium length, posterium diameter, and ratio of posterium length to trunk length (Table IV). Every population of *P. yongei* was compard with every other *P. yongei* population, a total of 21 comparisons; the *S. clenchi* population was compared with each of the 7 *P. yongei* populations. For each comparison between two populations, the absolute values of the differences between the five pairs of mean character values were summed and averaged, each mean character value having first been reduced by setting the maximum value for that character at 100.

The equation used for mean character difference by Sneath and Sokal (1973) is

$$\frac{1}{n}\sum_{i=1}^{n}|X_{ij} - X_{ik}|$$

where n = number of characters and X_{ij}, X_{ik} = mean values of populations j, k for character i. The lower the mean character difference is between two populations, the more similar they are.

The resulting six mean character differences (M.C.D.) for each population of *P. yongei* were summed to give an overall value (Table V, Total M.C.D.). A comparison of these summed M.C.D.'s indicates that the total for any one population is not strikingly different from that of any other, although the range in values are rather large. The highest value for summed M.C.D.'s (67.7) occurs at one end of the depth range,

TABLE IV

Mean value, standard deviation, and sample number for five characters in seven populations of Prochaetoderma yongei *and one population of* Spathoderma clenchi

Station and depth	Trunk length mm			Posterium length mm			Trunk diameter mm			Posterium diameter mm			Posterium length: Trunk length		
	$\bar{X}$	S.D.	(n)	$\bar{X}$	S.D.	(n)	$\bar{X}$	S.D.	(n)	$\bar{X}$	S.D.	(n)	$\bar{X}$	S.D.	(n)
Prochaetoderma yongei															
No. American B.															
CH-88 Sta. 207 805-811 m	1.11	.23	(70)	.36	.08	(70)	.37	.05	(70)	.22	.03	(70)	.33	.09	(70)
CH-50 Sta. 87 1102 m	1.38	.24	(35)	.48	.12	(35)	.34	.07	(36)	.19	.02	(36)	.35	.06	(35)
AII-30 Sta. 128 1254 m	1.39	.23	(44)	.43	.08	(44)	.33	.05	(42)	.21	.03	(44)	.31	.04	(44)
AII-12 Sta. 73 1470–1330 m	1.55	.27	(137)	.54	.11	(137)	.35	.06	(136)	.19	.03	(133)	.35	.06	(137)
CH-88 Sta. 209 1501–1693 m	1.16	.30	(46)	.38	.09	(46)	.31	.07	(44)	.21	.03	(46)	.34	.05	(46)
AII-30 Sta. 131 2178 m	1.61	.25	(15)	.53	.17	(15)	.33	.07	(15)	.21	.04	(15)	.33	.08	(15)
Namibia B.															
AII-42 Sta. 191 1600 m	1.39	.19	(43)	.41	.09	(43)	.38	.05	(43)	.22	.03	(43)	.30	.05	(43)
Spathoderma clenchi															
No. American B.															
AII-30 Sta. 131 2178 m	1.01	.22	(35)	.51	.14	(35)	.35	.05	(37)	.22	.03	(32)	.51	.12	(35)

that is, in the population from the shallowest station; the lowest value (45.0) occurs in one of the populations at mid-depth range. The values for both total and range in M.C.D.'s in the population geographically most distant from the others (Namibia Basin) fall well within the values for other populations.

High total M.C.D. values, such as 67.7 for the short, stubby forms at the shallow station (Sta. 207) and 65.0 for the long, slender forms at the type locality at mid-depth range (Sta. 73), do not contain any taxonomic information on how these populations differ (Cain and Harrison, 1958); however, an examination of actual measurements (Table IV) indicates that in these two populations, differences in length are responsible for the high total M.C.D. values.

Body shape in specimens of *P. yongei* and *Spathoderma clenchi* is often so similar that species determination was always based on spicule morphology. However, the average M.C.D. value of the seven comparisons between the single *S. clenchi* population

TABLE V

Total, range and average mean character differences (M.C.D.'s) between seven populations of Prochaetoderma yongei, *and range and average M.C.D.'s between one population of* Spathoderma clenchi *and the seven* P. yongei *populations*

Station	Depth m	Total M.C.D.*	Range of M.C.D.'s	Average M.C.D.
		Prochaetoderma yongei		
No. American B.				
CH-88 Sta. 207	805–811	67.7	5.8–16.7	11.3
CH-50 Sta. 87	1102	51.2	4.9–12.9	8.5
AII-30 Sta. 128	1254	45.0	4.7–10.5	7.5
AII-12 Sta. 73	1470–1330	65.0	4.8–16.7	10.8
CH-88 Sta. 209	1501–1693	60.7	5.8–15.1	10.1
AII-30 Sta. 131	2178	59.8	4.8–15.5	10.0
Namibia B.				
AII-42 Sta. 191	1600	56.3	4.7–13.1	9.4
		Spathoderma clenchi		
No. American B.				
AII-30 Sta. 131	2178	**	14.9–19.7	17.2

* See text for explanation.

** Total M.C.D.'s between *S. clenchi* and *P. yongei* are meaningless.

and the seven *P. yongei* populations is 17.2, much higher than the average values—7.5 to 11.3—among *P. yongei* populations, although there is considerable overlap in the ranges of values in the two species. Thus mean character difference seems to be a sensitive measure of similarity in body shape within a species and of dissimilarity between two species.

The only character that is significantly different in all comparisons between the two species is the ratio of posterium length to trunk length (*t*-test of the means, $P < .001$ for all populations), and it is this character difference which accounts for the high M.C.D. values in *S. clenchi.* In contrast, the shallowest population of *P. yongei* was not significantly different from any other population of *P. yongei* in mean posterium- to trunk-length ratio, although it is the population with the highest value for total M.C.D. and the appearance of being most different in body shape. On the other hand, this ratio is not a perfect species indicator, for among all 7 *P. yongei* populations it was significantly different in 5 out of the total 21 comparisons.

It is concluded that (1) there is not sufficient difference in body shape, as quantified by mean character difference, among populations of *P. yongei* to consider that any of them belong to a different species; and (2) the ratio of posterium length to trunk length is an important taxonomic character in species of Prochaetodermatidae.

Distribution

Prochaetoderma yongei is very widely distributed on the continental slope between 800 and 2000 m in the northwestern and eastern Atlantic (Table I; Fig. 1, solid circles). It does not occur in samples taken between Cape Hatteras and the Argentine Basin in the western Atlantic or from depths less than 450 or greater than 2200 m. The samples in which it was taken in the eastern Atlantic are distributed at very great but rather even distances between 58°N and 23°S.

In the North American Basin, *P. yongei* is very abundant in both quantitative and nonquantitative samples; in the eastern Atlantic it is up to a hundredfold less abundant in sled trawls and was not taken in quantitative samples (Tables I, VII). Between depths of 1300 and 2100 m in both the northwestern and northeastern Atlantic *P. yongei* occurs sympatrically with the confamilial species *Spathoderma clenchi* (*q.v.*). In samples containing both species, *P. yongei* is the more abundant at depths less than 1800 m in the western Atlantic and 1900 m in the eastern Atlantic. (Anchor dredge samples are disregarded because of their inherent sampling bias.)

A single specimen of *P. yongei* taken from 530 m off Gay Head is 2.8 mm long, large for the species. A single specimen from 5042 m (CH-50 Sta. 81) is probably mislabeled.

Specimens examined

A total of 3122 specimens was examined, 2987 from the western Atlantic and 135 from the eastern Atlantic (Table I).

Genus *Spathoderma* n.g.

With characters of the family. Spicules spatulate, with flat base and relatively long, narrow blade rounded in cross-section, bent outward from body.

Distribution (based in part on species to be described): Atlantic continental slopes and abyssal plains.

From *spatha* (L.), a spatula.

Type species: *Spathoderma clenchi* n. sp., North American Basin.

Spathoderma clenchi n. sp.

Figs. 1, 3D–G, 4D, F, 9, 14 c^1, c^2; Tables I, III, IV, V, VII

Prochaetoderma sp. *c.* Scheltema, 1981, *Malacologia* **20:** 363.

Prochaetoderma sp. A. Rowe, Polloni, and Haedrich, 1982, *Deep Sea Res.* **29:** Table 2.

Prochaetoderma sp. A. Scheltema, 1985, *in* L. Laubier and C. Monniot, eds., *Peuplements Profonds du Golfe de Gascogne: Campagnes BIOGAS,* IFREMER, Brest, pp. 391–396, Tables 1, 2, 3.

Diagnosis: Opaque, less than 2½ mm long, posterium ⅓ total length; oral-shield spicules indistinct; spicules bent slightly outwards and oriented parallel to long axis of body, without ornamentation, greatest length 232 μm, blades narrow with concave sides, bent sharply outwards, base with parallel sides; greatest jaw length 435 μm; greatest tooth length 100 μm; central radula plate long and relatively narrow, greatest length 45 μm.

This species is named in honor of Dr. William J. Clench, who first encouraged me to study mollusks.

Holotype: North American Basin, 39°38.5′N, 70°36.5′W, 2178 m (ATLANTIS II-30 Sta. 131, 18/XII/66). USNM No. 850209.

Illustrated paratypes:

Nos. 1, 2: Type locality. USNM Nos. 850210 (No. 1), 850211 (No. 2).

No. 3: West European Basin, 57°59.7′N, 10°39.8′W, 2091 m (INCAL DS-01, 15/VII/76). MNHN, Paris.

No. 4: West European Basin, 55°07.7′N, 12°52.6′W, 2897 m (INCAL DS-09, 20/VII/76). MNHN, Paris.

Description

External morphology. Spathoderma clenchi is a small, opaque species with bent spicules which extend out from the body most noticeably at the junction of trunk and posterium; they are oriented parallel to the long axis of the body except ventrally, where they diverge along the midline (Fig. 3D–F). Average total body length at the type locality is 1.5 mm; greatest length is 2.2 mm. Both average and greatest trunk diameter are 0.4 mm. Posterium length is about ⅓ total length. Mean posterium length is 0.5 mm, and greatest length 0.9 mm; posterium diameter averages 0.2 mm and greatest diameter is 0.3 mm. The mean index of posterium to trunk length is 0.51, ranging from 0.27 to 0.87. The margin of the cloaca in lateral view is oblique. The oral shield is slightly larger than in *Prochaetoderma yongei* (Fig. 3G); oral shield spicules are present but not obvious.

Holotype: Total length 2.1 mm, trunk 1.4 mm by 0.4 mm, posterium 0.7 mm by 0.2 mm, index of posterium to trunk 0.50. Oral shield 0.06 by 0.12 mm; index of oral shield to trunk diameter 1.80.

Spicules. The spicules of *S. clenchi* lack ornamentation except for faint ridges on the blade seen only by scanning electron microscopy and an occasional subdued keel (Figs. 4D, F; 9). The sides of the base are straight and usually parallel; proximally the base is rounded or broadly triangular. In some spicules the base widens into a bulge at the waist. The sides of the blade are concave proximally at the waist where the blade rapidly narrows, except in spicules from region a_1 and ventral region b_2 where the blade may have convex sides.

The blade is narrow and sharply or roundly pointed. The isochromes are symmetrical. The thickest part of the spicule is at or just proximal to the waist in spicules from region b_1; further posteriorly along the body, the thickest part is the blade. The width of the base is usually narrower in spicules from the posterium (region *d*) than in those from the trunk. In lateral view the blade is bent sharply outwards, except in region *a* and in ventral region b_2 where the blade is bent towards the body. Spicules from the ventral side of the body are smaller than those from the dorsal side. A few short, thin spicules with a short distal point are scattered on the body.

Ranges in dimensions of spicules from region *c* are given for four populations in three basins in Table III.

Radula. The jaws and teeth, examined in nine specimens, are large and typical for the family (Fig. 14 c^1, c^2). Tooth length ranges up to 100 μm, and jaw length and width up to 435 and 151 μm, respectively. The radula has 8 to 10 rows of teeth. The central radula plate is curved and long, up to 45 μm, and slender, up to 6 μm wide, with the ends rounded or somewhat pointed.

Distribution

Spathoderma clenchi has the same geographic range as *Prochaetoderma yongei:* from the northwestern to the northeastern Atlantic and south to the Namibia Basin in the eastern Atlantic; like *P. yongei* it is not found south of Cape Hatteras in the western Atlantic (Fig. 1, triangles). In vertical range, the two species overlap on the continental slope, but *S. clenchi* ranges deeper onto the abyssal rise, down to 3356 m

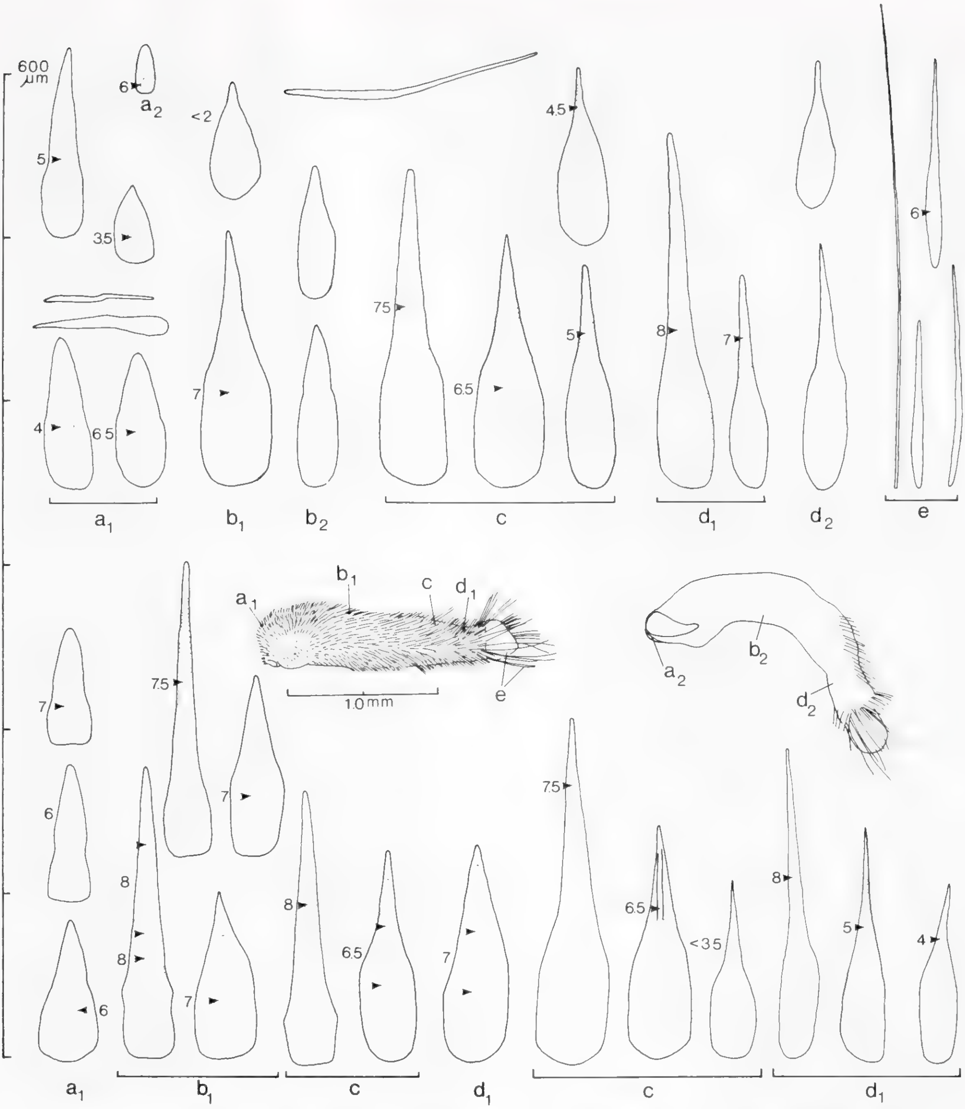
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FIGURE 9. Spicules of *Spathoderma clenchi* n.g. n. sp. *Upper row:* a_1–*e* from paratypes no. 1, left specimen, and no. 2, right specimen, 2178 m, North American Basin (type locality). *Lower row:* a_1–d_1 at left, spicules from several specimens from 1470 m, North American Basin (A II-12 Sta. 73);*c* and d_1 at right, spicules from paratype no. 3, 2091 m, West European Basin (region d_1 spicules somewhat oblique). Uppermost center spicule, lateral view showing blade (to right) bent outwards away from body; lateral views of a_1 spicules, to left, are bent somewhat towards body.

(Table I). It is commonly found in samples taken at depths over 2000 m, and is sometimes abundant (Table VII).

Specimens examined

A total of 1480 specimens was examined, 682 specimens from the western Atlantic and 798 from the eastern Atlantic (Table I).

Genus *Chevroderma* n. g.

With characters of the family. Spicules asymmetrical, base of spicules long, with a longitudinal groove and regularly spaced, chevron-shaped cross grooves reflecting crossed fibers of cuticle (Fig. 5F). Spicules of trunk flat-lying and arranged obliquely, diverging on each side of ventral midline, spiralling up dorsally and posteriorly, and meeting at a slight or pronounced angle along dorsal midline (Fig. 3). Thickest part of spicule is dorsal to longitudinal groove, overlapping ventral, thin side of next adjacent spicule dorsal to it (Fig. 10, upper left).
Distribution: Lower continental slopes and abyssal plains of the Atlantic and Pacific; Aleutian Trench.

From *chevron* (Fr.), a chevron.
Type species: *Chevroderma turnerae* n. sp.

Chevroderma turnerae n. sp.
Figs. 1, 3 L-P, 5 B, D, F, 10, 15 t^1–t^3; Tables I, VI, VII

Prochaetoderma sp. C. Scheltema, 1985, *in* L. Laubier and C. Monniot eds., *Peuplements Profonds du Golfe de Gascogne: Campagnes BIOGAS,* IFREMER, Brest, pp. 391–396, Tables 1, 2, 3.

Diagnosis: Large, opaque, up to 5½ mm in length and 0.8 mm in diameter, with a long posterium ⅖ or more total length; large oral shield and prominent oral shield spicules; spicules converge at pronounced angle along dorsal midline; trunk spicules up to 300 μm with long base and wide, short blade bluntly pointed, thickened proximal to waist; radula and jaws large, teeth up to 140 μm long, jaws up to 700 μm long, and central plate long, up to 50 μm, narrow, and curved.

This species is named in honor of Prof. Ruth D. Turner, who has given me many years of encouragement and advice.

Holotype: North American Basin, 35°50.0′N, 64°57.5′W, 4833 m (ATLANTIS II-24 Sta. 122, 21/VIII/66). USNM No. 850213.

Illustrated paratypes:

Nos. 1, 3: Type locality. USNM Nos. 850214 (No. 1), 850216 (No. 3).
No. 2: Angola Basin, 14°49′S, 9°56′E, 3797 m (ATLANTIS II-42 Sta. 195, 19/V/68). USNM No. 850217.
No. 4: Brazil Basin, 00°46.0′S, 29°28.0′W, 3459 m (ATLANTIS II-31 Sta. 156, 14/II/67). USNM No. 850219.
No. 5: West European Basin, 47°32′N, 9°35.9′W, 4237 m (BIOGAS-VI CP-14, 23/X/74). MNHN, Paris.
No. 6: Canary Basin, 27°14.9′N, 15°36.3′W, 2988 m (DISCOVERY 6711, 19/III/68). USNM No. 850221.
No. 7: Argentine Basin, 37°13.3′S, 52°45.0′W, 3305 m (ATLANTIS II-60 Sta. 259, 26/III/71). USNM No. 850223.

Description

External morphology. Chevroderma turnerae is large for a prochaetodermatid species with total body length averaging from 2.8 to 3.8 mm in three widely separated populations and ranging up to 5½ mm (Fig. 3L). Trunk diameter averages 0.5 mm, with greatest diameter 0.8 mm (Table VI). The posterium is long, from ⅖ to nearly

TABLE VI

Mean value, standard deviation, and sample number for five characters in four species of Chevroderma (C. turnerae, C. gauson, C. scalpellum, *and* C. whitlatchi)

Station and depth	Trunk length mm			Posterium length mm			Trunk diameter mm			Posterium diameter mm			Posterium length: Trunk length		
	X̄	S.D.	(n)	X̄	S.D.	(n)	X̄	S.D.	(n)	X̄	S.D.	(n)	X̄	S.D.	(n)
Chevroderma turnerae															
No. Amer. B.															
AII-24 Sta. 122 4833 m	1.9	.84	(11)	1.6	.68	(10)	0.5	.13	(11)	0.3	.09	(11)	.71	.20	(10)
W. European B.															
NOR. 113-EO13 4760 m	2.0	.72	(43)	1.6	.72	(43)	0.5	.13	(45)	0.3	.05	(45)	.80	.23	(43)
Argentine B.															
AII-60 Sta. 245 (subsample) 2707 m	1.7	.50	(16)	1.1	.40	(16)	0.5	.12	(16)	0.3	.09	(16)	.69	.21	(16)
C. gauson															
W. European B.															
CH-106 Sta. 330 4632 m	1.7	.52	(8)	0.8	.21	(8)	0.6	.14	(8)	0.3	.07	(8)	.52	.12	(8)
C. scalpellum															
Angola B.															
AII-42 Sta. 202 1427 m	1.0	.27	(50)	0.5	.15	(50)	0.4	.08	(50)	0.3	.05	(50)	.52	.12	(50)
C. whitlatchi															
Aleutian Trench															
SEVENTOW H-39 7298 m	1.0	.43	(31)	0.6	.36	(31)	0.3	.02	(31)	0.2	.06	(31)	.63	.14	(31)
Panama B.															
ALVIN dives 3912 m	0.9	.35	(31)	0.6	.19	(31)	0.3	.08	(31)	0.2	.05	(31)	.71	.26	(31)

½ body length; it averages 1.1 to 1.6 mm in length in three populations, with greatest length 2.9 mm, and 0.3 mm in diameter, with greatest diameter 0.5 mm. The posterium to trunk ratio averages 0.69 to 0.80, but the range is very large, from 0.32 to 1.36. The spicules of the trunk meet at a distinct angle along the dorsal midline (Fig. 3N).

The long spicule blades of the posterium extend out from the body. There are two rows of prominent oral shield spicules; the oral shield is distinctively large (Fig. 3O, P). The margin of the cloaca in lateral view is slanted.

Holotype: Total length 4.7 mm, trunk 2.8 by 0.7 mm; posterium 1.9 by 0.4 mm; index of posterium to trunk 0.68. Oral shield 0.14 by 0.17 mm; index of oral shield to trunk diameter 3.40.

Spicules. Trunk spicules are thick, with a long base relative to a short, wide blade that tapers to a rounded point (Figs. 5B, D, and 10, *b, c*). Greatest length is about 300 μm. The base is slightly rotated about its long axis. Greatest thickness, up to 8.5 μm, is about midway lengthwise, proximal to the waist. The sides of the base are somewhat convex, with the dorsal edge curved more than the ventral edge; the proximal end is rounded to somewhat pointed. The waist is either distinct or indistinct. The longitudinal groove usually runs the entire length of the base and often onto the blade. The blade is straight, or bent slightly outward. Spicules from the ventral side of the trunk are shorter but not narrower than spicules from the lateral and dorsal sides; the waist is not distinct (Fig. 10 b_2, c_2).

Oral shield spicules are large and thick (Fig. 10a_2); spicules at the junction of anterium and trunk (a_1) are thick and symmetrical. Spicules of the posterium (*d*) are more nearly symmetrical than those of the trunk; some are quite narrow and thickest at the waist, similar to spicules of *C. gauson* n. sp. (*q.v.*). Trunk spicules of immature specimens are sharply pointed (Fig. 10B left).

Faint ridges occur on some or all of the trunk spicules in populations other than those from the North American Basin, which lack ridges. At least some spicules from the posterium are ridged in every population (ridges not illustrated).

Radula: The teeth and jaws from ten specimens were examined; they are large and typical for the family (Fig. 15 t^1–t^3). Teeth range up to 140 μm in length; jaws range up to 700 μm in length and 300 μm in width, but are usually between 500 and 600 μm in length and between 200 and 250 μm in width. The central plate is long, up to 50 μm, relatively narrow, up to 10 μm, curved, and tapered at the ends. The brush membrane and the wing may be thickened, or tanned, in distal teeth.

Morphological variation

Greatest geographic variations in spicule morphology are in base width and degree of asymmetry. Spicules from specimens from the North American and deepest West European Basin have narrow bases relative to most other populations (Fig. 10, upper row and W spicules 1, 5). Wide-based spicules occur along with narrow-based spicules in the populations of the Brazil and Argentine Basins and the West European Basin (Fig. 10B, A, W spicules 3, 4). Spicules are all relatively wide-based from specimens taken from the Canary Basin southwards in the eastern Atlantic (Fig. 10C, An, N).

Most samples of *Chevroderma turnerae* are either too small for statistical analysis of body shape (see Table I) or comprised of mostly immature specimens. Samples from three widely separated geographic regions—North American Basin (ATLANTIS II-24 Sta. 122), West European Basin (NORATLANTE 113-E013), and Argentine Basin (ATLANTIS II-60 Sta. 245)—show no significant difference between means of five body measurements among the three populations except in posterium length (Table VI). In the Argentine Basin population, posterium length is statistically—and noticeably—shorter than in the other two populations. However, the ratio of posterium length to trunk length is not significantly different among the three populations.

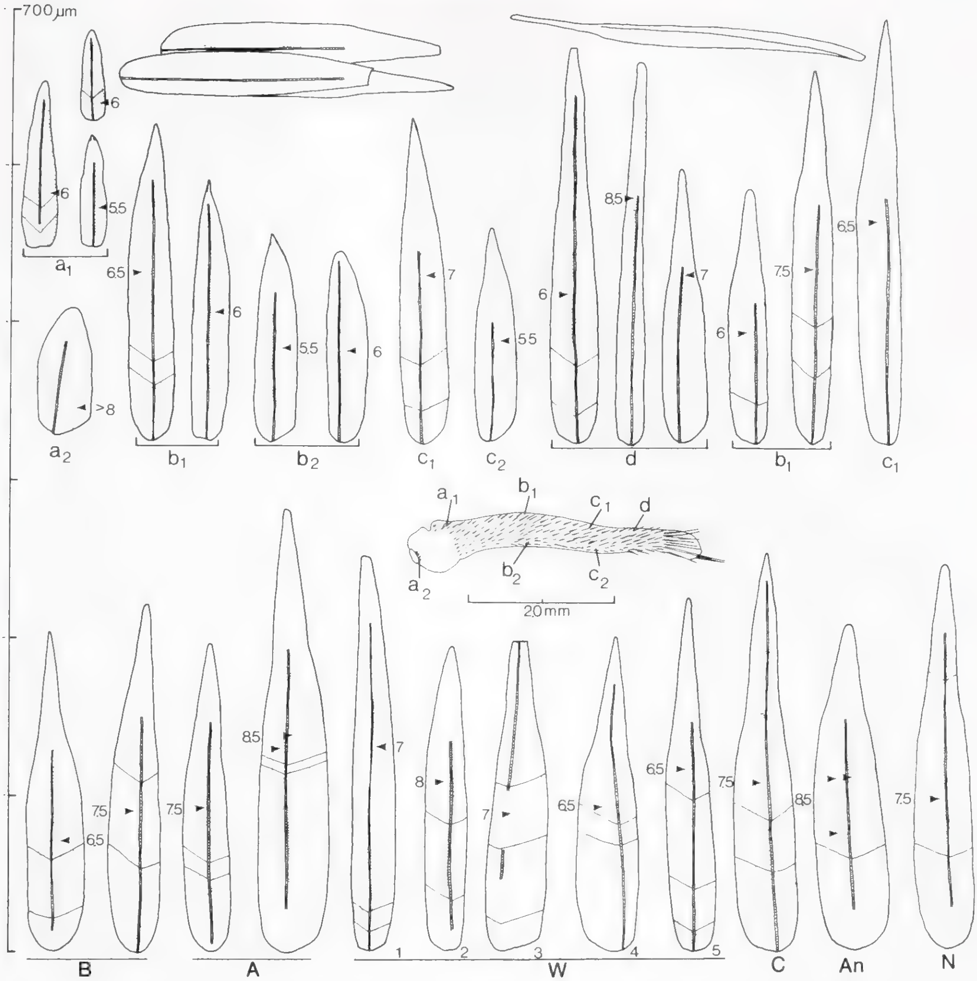

FIGURE 10. Spicules of *Chevroderma turnerae* n.g. n. sp. *Upper row:* a_1–d at left, paratype no. 1 (figured specimen), and b_1, c_1 at right, paratype no. 3, 4833 m, North American Basin (type locality); upper left, overlapped spicules showing *in situ* arrangement, anterior end to left, dorsal side uppermost; upper right, lateral view showing rotation and outward bend of short blade. *Lower row:* variation in morphology of spicules from trunk regions b_1 and c_1: B. immature specimen, paratype no. 4, 3459 m, Brazil Basin; A. two specimens from 3305 m, Argentine Basin, right spicule paratype no. 7; W. several specimens from West European Basin: spicule 1 from a deep station at 4823 m (INCAL CP-10), spicules 2 and 3 from shallower stations at 2897 and 2634 m (INCAL DS-09 and OS-01), spicules 4 and 5 from 4237 and 4706 m at stations further east in the Bay of Biscay (paratype no. 5 and BIOGAS VI CP-17); C. paratype no. 6, 2988 m, Canary Basin; An. paratype no. 2, 3797 m, Angola Basin; N. immature specimen, 4184 m, Namibia Basin (WALDA DS-04).

The larger size, but not the shape, of spicules also differentiates the shallower Argentine Basin population of *C. turnerae* from the other two populations (Fig. 10A).

Jaw length in one of two Argentine Basin specimens is great (700 µm) compared to jaw length in five specimens from three other widely separated populations (493–580 µm, North American, West European, and Angola Basins). However, jaw length in the second Argentine Basin specimen (560 μm) falls within the limits measured in the other three populations.

On the bases of body measurements and spicule and radula morphology, the Argentine Basin continental slope populations of *C. turnerae* are considered to be small, short-tailed members of the species.

Distribution

Chevroderma turnerae is a cosmopolitan abyssal species of the Atlantic basins, absent only in samples from the Guyana and Iberian Basins (Table I; Fig. 1, open circles). Its depth range is also great, from a little over 2100 m to 5208 m. It is most commonly found at depths greater than 3000 m except in the Argentine Basin, where it was taken in largest numbers at depths less than 3000 m.

Chevroderma turnerae has never been taken in large numbers at any one locality, even with an epibenthic sled trawl; the greatest densities sampled quantitatively were in the Bay of Biscay (Tables I, VII).

Material examined

Five hundred thirty-eight specimens were examined, 222 from the western Atlantic and 316 from the eastern Atlantic.

Chevroderma gauson n. sp.

Figs. 1, 3Q–T, 5A, 11, 15 g^1, g^2; Tables I, VI

Prochaetoderma sp. D. Scheltema, 1985, *in* L. Laubier and C. Monniot, eds., *Peuplements Profonds du Golfe de Gascogne: Campagnes BIOGAS,* IFREMER, Brest, pp. 391–396, Tables 1, 2.

Diagnosis: Opaque, broad, with very long spicules distinctly bent outward from body; greatest body length 3.6 mm, greatest diameter 0.8 mm, posterium ⅓ total length and broad; oral shield spicules small, indistinct; trunk spicules up to nearly 500 μm, thickest at distinct waist, blade long, narrow, and bent outwards, longitudinal groove distinct to faint; radula large, teeth up to about 130 μm in length, jaws up to 626 μm, central plate long, up to 48 μm, wide and thick with a shallow groove, ends blunt.

The species name means "bent outwards."

Holotype: West European Basin, 50°43.5′N, 17°51.7′W, 4632 m (CHAIN-106 Sta. 330, 24/VIII/72). USNM No. 850226.

Illustrated paratypes:

No. 1: West European Basin, 50°04.7′N, 15°44.8′W, 4426 m (CHAIN-106 Sta. 328, 23/VIII/72). USNM No. 850229.
No. 2: Type locality. USNM. No. 850227.
No. 4: West European Basin, 48°19.2′N, 15°15.9′W, 4829 m (INCAL 0S-02, 2/VIII/76). MNHN, Paris.
No. 5: West European Basin, 48°19.2′N, 15°23.3′W, 4829 m (INCAL WS-03, 1/VIII/76). MNHN, Paris.

Description

External morphology. Chevroderma gauson is a moderately large, broad, opaque species with very long spicules which are bent outward and spiral upwards and pos-

TABLE VII

Vertical distribution, average sample number, and greatest density of three Prochaetodermatidae species in the North American and West European Basins

	Samples (N)*		No. Amer. B.				W. Europ. B.			
Depth m	No. Amer. B.	W. Europ. B.	Samples with species	No. individ. $(\Sigma\bar{X})$	$\Sigma X/N = \bar{X}$	$\frac{s^2_{\bar{x}}}{\bar{X}}$	Samples with species	No. individ. $(\Sigma\bar{X})$	$\Sigma X/N = \bar{X}$	$\frac{s^2_{\bar{x}}}{\bar{X}}$
				Prochaetoderma yongei						
0–500	6	3	1	3	0.5	3.0	0	—	—	—
501–1000	2	6	2	167	83.5	163.0	0	—	—	—
1001–1500	3	4	3	1942	647.3	422.5	1	19	4.8	18.8
1501–2000	1	14	1	435	435.0	—	5	17	1.2	5.3
2001–2500	5	45	4	281	56.2	95.2	3	8	0.2	3.2
2501–3000	6	23	0	—	—	—	0	—	—	—
3001–3500	1	6	0	—	—	—	0	—	—	—
3501–4000	7	5	0	—	—	—	0	—	—	—
4001–4500	1	43	0	—	—	—	0	—	—	—
4501–5000	22	28	0	—	—	—	0	—	—	—
>5000	4	0	0	—	—	—	—	—	—	—
Greatest density sampled**:				400 m^{-2}				n.d.		
				Spathoderma clenchi						
0–500	6	3	0	—	—	—	0	—	—	—
501–1000	2	6	0	—	—	—	0	—	—	—
1001–1500	3	4	1	19	6.3	19.1	0	—	—	—
1501–2000	1	14	1	6	6.0	—	1	2	0.1	2.9
2001–2500	5	45	5	579	115.8	125.1	14	417	9.3	154.0
2501–3000	6	23	0	—	—	—	10	342	14.9	102.4
3001–3500	1	6	1	1	1.0	—	1	10	1.7	9.8
3501–4000	7	5	0	—	—	—	0	—	—	—
4001–4500	1	43	0	—	—	—	0	—	—	—
4501–5000	22	28	0	—	—	—	0	—	—	—
>5000	4	0	0	—	—	—	—	—	—	—
Greatest density sampled**:				275 m^{-2}				8 m^{-2}		
				Chevroderma turnerae						
0–500	6	3	0	—	—	—	0	—	—	—
501–1000	2	6	0	—	—	—	0	—	—	—
1001–1500	3	4	0	—	—	—	0	—	—	—
1501–2000	1	14	0	—	—	—	0	—	—	—
2001–2500	5	45	0	—	—	—	1	1	0.0	—
2501–3000	6	23	0	—	—	—	4	4	0.2	—
3001–3500	1	6	1	1	1.0	—	2	12	2.0	4.8
3501–4000	7	5	3	14	2.0	3.3	2	13	2.6	8.8
4001–4500	1	43	1	14	14.0	—	23	108	2.5	8.8
4501–5000	22	28	7	59	2.7	8.4	16	63	2.3	3.3
>5000	4	0	1	2	0.5	2.0	—	—	—	—
Greatest density sampled**:				8 m^{-2}				24 m^{-2}		

* Sanders sled trawl, "Chalut à perche," Oban sled, and Wormley trawl.

** Spade box corer, Birge-Ekman box corer.

teriorly in broken diagonals (Fig. 3Q). Mid-dorsally the spicules are arranged nearly parallel to the long axis of the body (Fig. 3S). Total body length averages 2.5 mm in one population; greatest length is 3.6 mm (Table VI). The broad trunk averages 0.6 mm and ranges up to 0.8 mm in diameter. The posterium is one-third total length, and broad, 0.3 mm average. Posterium length averages 0.8 mm, with posterium to trunk length index averaging 0.52 but ranging widely, from 0.38 to 1.14. The oral shield spicules are small and indistinct beside a medium-size oral shield (Fig. 3T).

Holotype: Total length 2.6 mm; trunk 1.6 by 0.7 mm; posterium 1.0 by 0.4 mm; index of posterium to trunk length 0.62. Oral shield 0.08 by 0.13 mm; index of oral shield to trunk diameter 1.49.

Spicules. Trunk spicules are very long and often narrow; a long, narrow blade tapers from a distinct waist to an acute or rounded apex (Figs. 5A, 11). The greatest thickness, up to 9 μm, is at the waist. Sides of the base are nearly parallel or slightly convex; the proximal end is usually triangular. The distinct or faint longitudinal groove seldom runs onto the blade. The blade is bent outward. Faint ridges often run along the base parallel to the longitudinal groove. The greatest length of the trunk spicules is nearly 500 μm. The trunk bears a few short, thin spicules with sharply pointed, short blades. Spicules from the ventral midline of the trunk are short, with a short blade and distinct waist (Fig. 11b_2). Spicules from the posterium (*d*) are similar to those of the trunk. Oral shield spicules (a_2) are similar to, but smaller than, those of

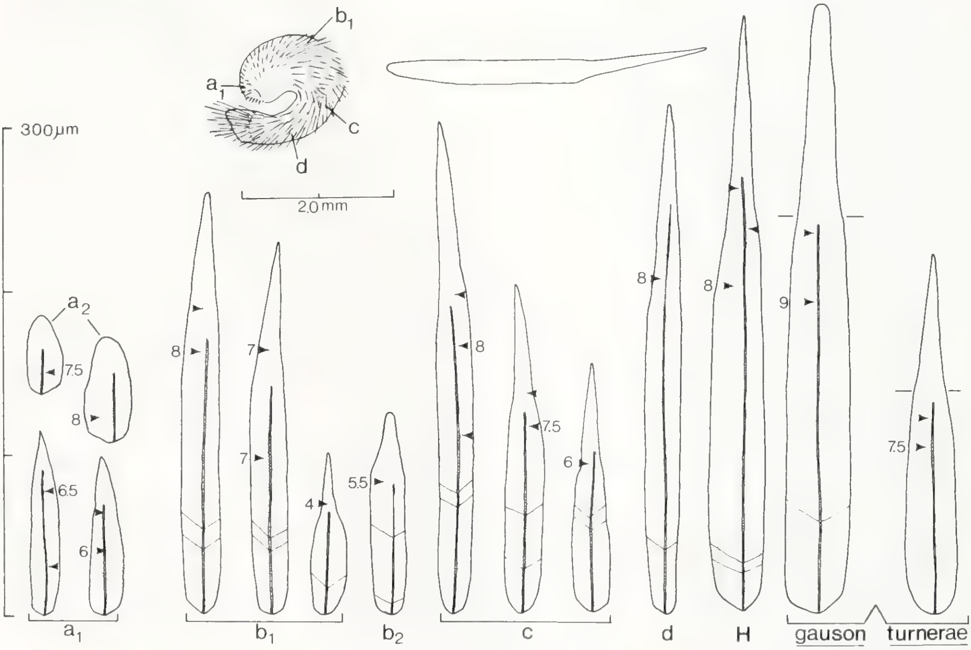

FIGURE 11. Spicules of *Chevroderma gauson* n. sp.: a_1–*d* and specimen, paratype no. 1, 4426 m, West European Basin; oral shield spicules a_2 from paratype no. 2, 4632 m, West European Basin (type locality), and b_2 ventral trunk spicule from paratype no. 4, 4829 m, West European Basin; H. holotype, trunk spicule. Far right: spicules from specimens of *C. gauson* and *C. turnerae,* both of about average size, taken in the same sample at the type locality.

C. turnerae; spicules at the junction of anterium and trunk (a_1) are similar in the two species.

Radula. The radula is typical for the family (Fig. 15 g^1, g^2). The teeth are probably large, up to perhaps 130 μm; however, exact measurements were not made because the tips of the posterior teeth from the three radulae examined were brittle and broke off in preparation, and the four anterior pairs of teeth were worn. The jaws are long, up to 626 μm, and up to 261 μm wide. The central plate is long, up 48 μm, wide, up to 10 μm, thick, and curved; it has a shallow groove running part or most of the length and the ends are blunt and rounded.

Differentiation from C. turnerae

Chevroderma gauson and *C. turnerae* were taken together in three out of the four stations where *C. gauson* occurred (Table I). Although the two species are distinctly different from each other in body shape where they co-occur, *C. gauson* is quite similar in shape to the short-tailed *C. turnerae* from the Argentine Basin continental slope and not significantly different from it in length or width of either trunk or posterium (Table VI). However, *C. gauson* is significantly different from all three *C. turnerae* populations analysed in the measure of posterium-to-trunk-length index ($P < .05$).

Spicules of *C. gauson* and *C. turnerae* (Fig. 11, right) are distinguished primarily by length, relationship of greatest thickness to waist, and angle of blade to base, and secondarily by convexity of sides of the base, width of base and blade, shape of the proximal end, and distinctness of the waist. If they are visible in a specimen, the very large oral shield spicules of *C. turnerae* are distinctive.

The central plates distinguish the radulae of the two species (Fig. 15): in *C. turnerae,* but not *C. gauson,* the ends are tapered, and in *C. gauson,* but not *C. turnerae,* the plate is thick and bears a groove.

Distribution

Chevroderma gauson has been taken only at abyssal depths greater than 4400 m in the northern West European Basin north of 48°N (Table I; Fig. 1, squares). It was not taken in the abyss of the Iberian Basin just to the south (cruise ABYPLAINE, MNHN, Paris).

Specimens examined

The description is based on a total of 19 specimens from four stations.

Chevroderma scalpellum n. sp.

Figs. 1, 3U-W, 5C, 12, 15s; Tables I, VI

Diagnosis: Short, broad, translucent, less than 2½ mm long and up to 0.6 mm in diameter, posterium one-third total length; oral shield and oral shield spicules thin, small, and very indistinct; spicules with broad base, short, narrow blade, and distinct waist, with longitudinal groove not reaching proximal end of base, greatest length less than 300 μm; radula of moderate size, tooth length up to 106 μm, jaw length up to 522 μm, central plate long, 43 μm, and narrow, 7 μm, with blunt ends.

The species name means "a little scalpel."

Holotype: Angola Basin, 9°05′S, 12°17′E, 1427 m (ATLANTIS II-42 Sta. 202, 23/V/68). USNM No. 850231.

Illustrated paratypes:

Nos. 1, 2: Type locality. USNM Nos. 850232 (No. 1), 850233 (No. 2).
No. 3: Cape Verde Basin, 10°30.0′N, 17°51.5′W, 1624 m (ATLANTIS II-31 Sta. 142, 5/II/67). USNM No. 850235.

Description

External morphology. Chevroderma scalpellum is a very small, broad species averaging 1.5 mm in length and 0.4 mm in trunk diameter in one population (Table VI); the largest specimen is 2.4 mm long, and diameter ranges up to 0.6 mm. The posterium is broad, 0.3 mm on average, and one-third total body length, averaging 0.52 in posterium to trunk index; posterium length averages 0.5 mm and ranges from 0.3 to 0.8 mm. Mid-dorsally the spicules are arranged nearly parallel to the long axis of the body, but meet at a distinct angle where the trunk joins the posterium (Fig. 3W). The oral shield is small and indistinct with a thin cuticle; oral shield spicules are also small and indistinct (Fig. 3V).

Holotype: Total length 1.7 mm; trunk 1.2 by 0.5 mm; posterium 0.5 by 0.3 mm; index of posterium to trunk length 0.42. Oral shield 0.06 by 0.09 mm; index of oral shield to trunk diameter 1.08.

Spicules. The spicule base is broad relative to a usually short, narrow, often sharply pointed blade bent slightly outward; the waist is conspicuous (Figs. 5C, 12). Many spicules are nearly symmetrical. The longitudinal groove runs only part way along the base, not extending to the proximal edge but often extending onto the blade; it is sometimes nearly or totally lacking. The base may have faint, rather broad ridges and

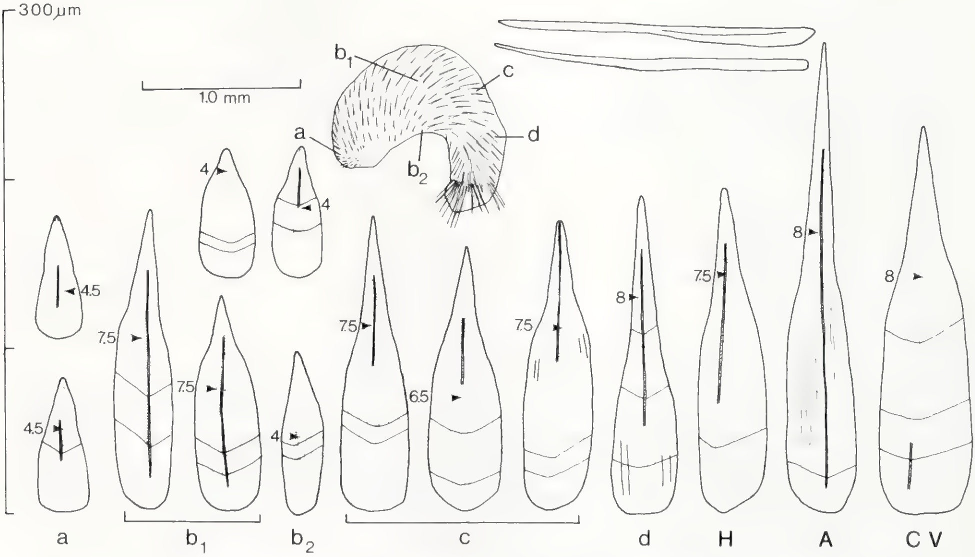

FIGURE 12. Spicules of *Chevroderma scalpellum* n. sp.: *a–d* and specimen, paratype no. 1, 1427 m, Angola Basin (type locality); H. holotype, trunk spicule; A. spicule from body region *c* of another specimen from type locality; CV. spicule from region *c*, paratype no. 3, 1624 m, Cape Verde Basin. Upper spicules: above, oblique view of body region *c* spicule; below, lateral view region *d* spicule; distal ends to left.

grooves; the sides of the base are straight to somewhat convex. Greatest thickness lies at the waist. Ventral spicules of the trunk (Fig. 12 b_2) are shorter than most other trunk spicules (b_1) and are thickened further proximally. Spicules of the anterium (a) have a broad base like those of the trunk. The bases of spicules from the posterium (d) are the same length or shorter than those from the trunk, and the blades are bent further outward. Most spicules from trunk regions b and c of Angola Basin specimens are no longer than 225 μm, but spicules from trunk region c in some specimens range up to nearly 300 μm (Fig. 12A). Spicules from Cape Verde Basin specimens are larger, both broader and longer, than those from most of the Angola Basin specimens (Fig. 12CV).

Radula. The radula, examined from two specimens, is of moderate size for the family (Fig. 15s). Greatest tooth length is 106 μm; jaw length and width are up to 522 and 215 μm, respectively. The central plate is long and narrow, up to 43 μm long by 7 μm wide, and has blunt, scarcely tapered ends.

Differentiation from other Chevroderma *species*

Chevroderma scalpellum is distinguished by its small size, indistinct oral shield, and spicules with their broad bases and short, narrow blades.

Distribution

Chevroderma scalpellum is an eastern Atlantic species found only in the Cape Verde and Angola Basins between about 10°N and 10°S over a narrow vertical range between 1427 and 2644 m (Table I; Fig. 1, hexagons).

Specimens examined

A total of 102 specimens from five samples was examined.

Chevroderma whitlatchi n. sp.

Figures 3H–K, 5E, 13, 15 w^1, w^2; Tables II, VI

Diagnosis. Usually small, average length about 1.5 mm but up to 4.2 mm, with long posterium ⅖ total length; may have conspicuous translucent hump where trunk joins posterium; oral shield spicules small, distinct; spicules converge at angle along dorsal midline; trunk spicules with long base, distinct waist, and short, abruptly tapered, sharply pointed and broadly keeled blade, up to 200 μm long; radula small, tooth with wing thickened and non-membranous, up to 100 μm long, jaws up to 320 μm long, central plate short with rounded ends.

This species is named for Dr. Robert B. Whitlatch, who gave me the Panama Basin material to examine.

Holotype: Panama Basin, 5°20.7′N, 81°56.2′W, 3912 m (ALVIN Dive 1239, Control 3, Core #2, 14/VI/82). USNM No. 850237.

Illustrated paratypes:

No. 1: Type locality (ALVIN 1232 Inj. BC #2). USNM No. 850238.
Nos. 2, 3: Aleutian Trench, 50°58.0′N, 171°37.5′W, 7298 m (SEVENTOW Leg 7, H-39, 20/VII/70). USNM Nos. 850240 (No. 2), 850241 (No. 3).

Description

External morphology. Chevroderma whitlatchi is small and opaque; in many contracted specimens, the trunk is broadest posteriorly, producing a translucent hump at the juncture with the posterium. Spicules of the anterior half of the trunk, when it is contracted, assume an upright position; further posteriorly they meet at a distinct angle with the tips overlapped along the dorsal midline (Fig. 3H). They are arranged parallel to the body along the dorsal side of the posterium. Total length averages 1.5 and 1.6 mm in two populations (Table VI), with greatest length 4.2 mm. Trunk diameter averages 0.3 mm and ranges up to 0.6 mm. The posterium is long, two-fifths total length, and averages 0.6 mm in length by 0.2 mm in diameter, with greatest dimensions 2.1 and 0.3 mm, respectively; posterium to trunk length index averages 0.63 and 0.71 in the two populations, with a large range, 0.36 to 1.33. The two populations were not significantly different in any of five body measurements (Table VI). The oral shield spicules are small but distinct; the oral shield is large (Fig. 3K).

Holotype: Total length 2.7 mm; trunk 1.6 by 0.4 mm; posterium 1.1 by 0.3 mm; index of posterium to trunk 0.69. Oral shield 0.08 by 0.17 mm; index of oral shield to trunk diameter 3.40.

Spicules: The blade of trunk spicules is short relative to the base and narrow, abruptly tapering from a distinct waist to a usually pointed apex (Figs. 5E, 13*c*, H, A). The blade is bent outwards from the body wall and usually bears a medial ridge (juvenile) or distinct broad keel. The base is narrow and rotated about the long axis. The sides of the base are usually nearly straight and tapered proximally, so that the base is widest at the waist. Trunk spicules are thickest at, or just distal to, the waist. The longitudinal groove seldom extends beyond the base and may be very faint. Greatest spicule length is about 200 μm. Spicules from the anterior trunk (a_1) are wide; those from the ventral side of the trunk (*b*) have a distinct waist. Spicules from the

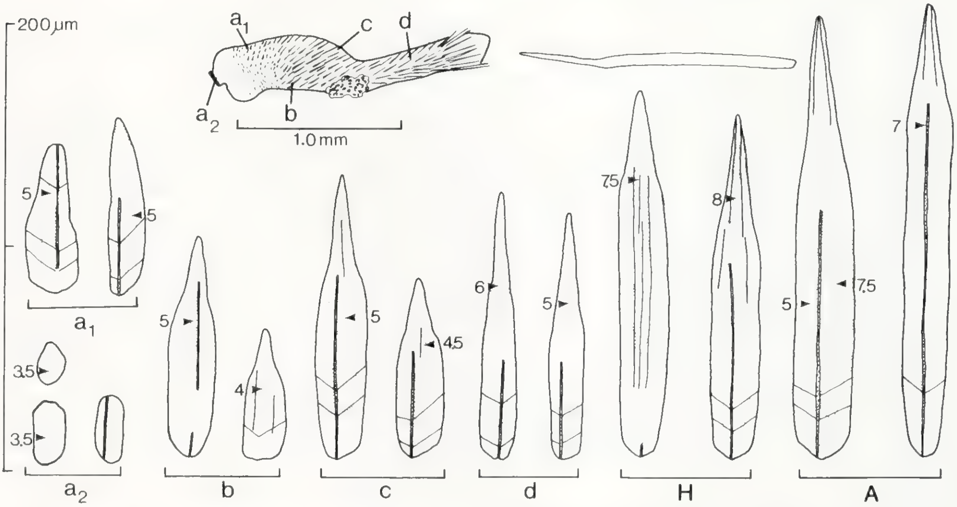

FIGURE 13. Spicules of *Chevroderma whitlatchi* n. sp.: a_1–*d* and specimen, paratype no. 1, juvenile, 3912 m, Panama Basin (type locality); H. holotype, body region *c;* A. region *c* spicules from two specimens, 7298 m, Aleutian Trench, right spicule from paratype no. 3; above, lateral view, distal end to left, showing outward bend of blade.

posterium (*d*) have a narrower base and longer blade than those from the trunk. The oral shield spicules (a_2) are small and thin.

Radula. The radula and jaws differ from other species of *Chevroderma* in their smaller size; in the thickened, twisted base of the jaws; in the serrations of the brush membrane, which are either very thin or lacking (although present in juvenile specimens); and in the thickened, non-membranous tooth wing (Fig. 15w^1, w^2). In four specimens examined, tooth length was 110 μm in a large specimen 3.3 mm long and jaw length and width 429 μm and 157 μm, respectively; in specimens of average length, tooth length is about 70 μm and jaw length and width range up to 320 and 110 μm, respectively. The central plate is short, up to 27 μm, and 5 to 7 μm wide, with usually rounded, untapered ends.

Distribution

Chevroderma whitlatchi is an abyssal and hadal species taken in the central and eastern North Pacific. It occurs at high densities in the soupy muds of the Panama Basin and Aleutian Trench. Like *C. turnerae,* it covers a great depth range, from

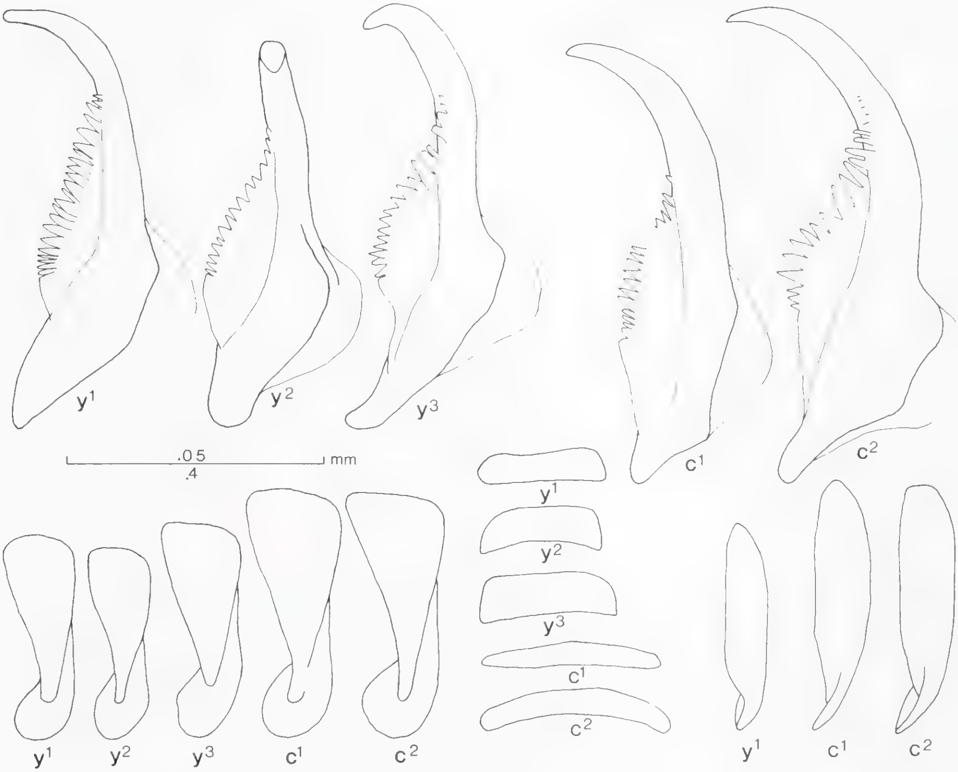

FIGURE 14. Radula teeth, central plates and jaws of *Prochaetoderma yongei* n. sp. and *Spathoderma clenchi* n.g. n. sp. y = *P. yongei:* y^1 paratype no. 1, 1470 m, North American Basin (type locality); y^2 paratype no. 3, 805 m, North American Basin; y^3 paratype no. 2, 1546 m, Namibia Basin; c = *S. clenchi:* c^1 paratype no. 2, 2178 m, North American Basin (type locality); c^2 paratype no. 4, 2897 m, West European Basin. Scale equals 0.05 mm for teeth and central plates and 0.4 mm for jaws.

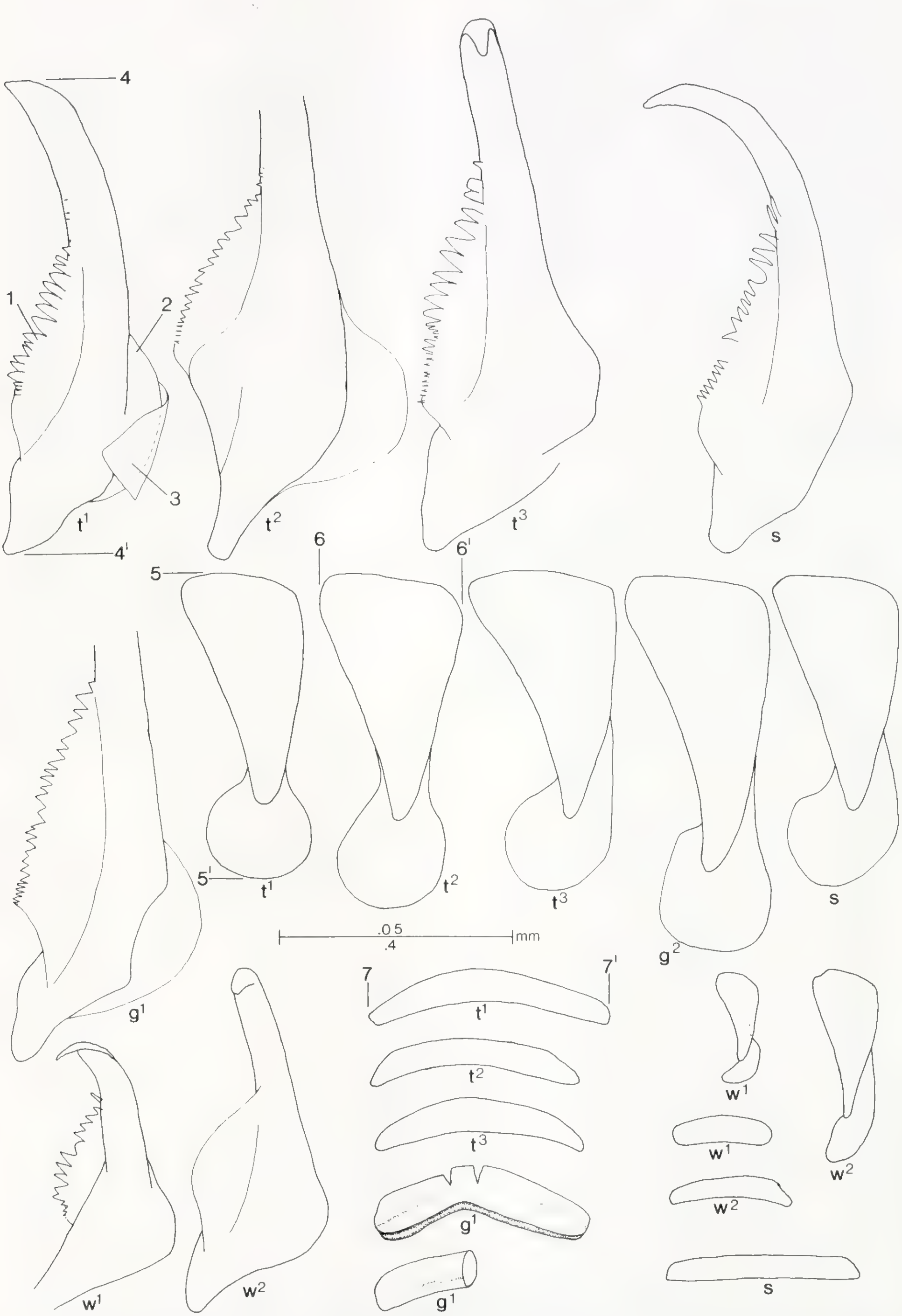
4
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FIGURE 15. Radula teeth, central plates and jaws of *Chevroderma* (n.g.) species. t = *C. turnerae* n. sp.: t^1 paratype no. 3, 4833 m, North American Basin (type locality); t^2 specimen from 3305 m, Argentine Basin (AII-60 Sta. 259); t^3 paratype no. 2, 3797 m, Angola Basin; s = *C. scalpellum* n. sp.: paratype no. 2,

2800 m off Oregon and 2727 m near the Galapagos Rift to over 5000 m in the mid-Pacific and over 7000 m in the Aleutian Trench.

About 90 percent of the specimens in box core samples from the Aleutian Trench and Panama Basin occurred within the upper 2 to 3 cm of sediment; the remaining 10 percent were below this level.

Material examined

Sixty-eight specimens were examined, 62 of them from the Panama Basin and Aleutian Trench, the remaining 6 scattered among 4 locations (Table II).

Discussion

Taxonomic characters

The dependence on spicule morphology for delimiting species and genera in the Prochaetodermatidae is based on ease of use, accessibility of the character, and biological considerations. Spicules and the cuticle in which they are embedded probably not only serve as a protective cover, but also—and perhaps primarily—as antagonists to muscle activity during locomotion. They also may adapt the animals to particular horizons within the sediment. Very small differences between species in body wall musculature, which would be very difficult to determine histologically, can be expected to be magnified in the morphology of the spicules. Body wall musculature, and thus spicule morphology, is considered to be a conservative character.

The body measurements of preserved, contracted specimens, although not descriptive of living animals, are related to the arrangement of internal organs and body wall musculature and allow for statistical analysis not readily possible with spicules, which vary greatly in size within a single specimen. As most identifications of deep-sea Aplacophora will be of preserved specimens, body measurements should be of continuing usefulness.

The variability in taxonomic characters described here and interpreted as belonging to single species may actually be due to the existence of sibling species living in different basins, but there is neither sufficient knowledge about the reproductive biology of these animals nor adequate samples on which to base judgements. It has seemed most sensible to treat apparently minor character differences between specimens from different ocean basins as variations within a species, rather than naming species according to their locality, *i.e.,* by ocean basins. A like situation in the protobranch family Malletiidae has recently been treated in a similar manner (Sanders and Allen, in press).

Distribution

Geographic. Vertical depth distribution and horizontal geographic range are directly related to one another in the Prochaetodermatidae. Three of the five Atlantic species described here—*Prochaetoderma yongei, Spathoderma clenchi,* and *Chevroderma turnerae*—have vertical depth ranges greater than 1500 m; all three are amphi-Atlantic

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1427 m, Angola Basin (type locality); g = *C. gauson* n. sp.: g^1 specimen from 4632 m, West European Basin (type locality); g^2 paratype no. 1, 4426 m, West European Basin; w = *C. whitlatchi:* w^1 paratype no. 1, 3912 m, Panama Basin (type locality); w^2 specimen from 7298 m, Aleutian Trench (SEVENTOW Leg 7 Sta. H-39). 1 membranous serrated brush, 2 membranous wing, 3 lateral toothlike projection of radula membrane, 4-4′ tooth length, 5-5′ jaw length, 6-6′ jaw width, 7-7′ central plate length. Scale = 0.05 mm for teeth and central plates, 0.4 mm for jaws.

and have been taken from four or more ocean basins (Fig. 1, Table I). One, *C. turnerae*, is an abyssal species; both *P. yongei* and *S. clenchi* are continental slope species that do not extend into the abyss below 3300 m. *Chevroderma scalpellum* and *C. gauson* have both restricted depth ranges and geographic distributions; the former, with a depth range of 1217 m, is restricted to two adjacent ocean basins, and the latter, with a depth range of 403 m, occurs only in the northern West European Basin.

Such a correspondence in range of vertical and horizontal distribution is not unique to the Prochaetodermatidae and is considered to be at least in part due to mode of development and dispersal ability (R. Scheltema, 1972; Sanders, 1977). For an example, the protobranch bivalves are a molluscan group similar to the Aplacophora in development insofar as it is known for the two groups; either a lecithotrophic larva develops within a ciliated, cellular test or development is direct. A compilation from studies on 27 protobranch species in six families or subfamilies in the Atlantic (Allen and Sanders, 1973, 1982; Sanders and Allen, 1973, 1977, in press) shows that fourteen protobranch species have vertical depth ranges less than 1500 m; all but one of these are restricted to one or two ocean basins and to one side of the Atlantic. The remaining 13 species have depth ranges greater than 1500 m and are all amphi-Atlantic or occur in more than one ocean; but unlike the Prochaetodermatidae with large depth ranges, all 13 extend into the abyss below 3300 m.

R. Scheltema (1972, Table II) showed that two out of seven species of the protobranch genus *Nucula* in the northwest Atlantic were abyssal and had depth ranges greater than 1500 m, geographic ranges in three amphi-Atlantic basins, and lecithotrophic development. In one species, however, a lecithotrophic larval stage was related to a restricted depth range (less than 1000 m) and a geographic range of only two amphi-Atlantic basins, so factors other than mode of development determine distribution. A detailed study of reproduction and development in the species of Prochaetodermatidae described here has not yet been made.

Species with a dispersal ability are assumed to be better able to become widespread in the continuous abyss than on the continental slopes. However, both *P. yongei* and *S. clenchi* are broadly distributed geographically despite their restriction to less than 2178 and 3356 m, respectively, although in the western Atlantic they do not breach the partial zoogeographic barrier at 34°N and are restricted to the North American Basin, probably because of slope currents (Cutler, 1975). The abyssal *C. turnerae*, on the other hand, is ubiquitous throughout Atlantic basins, apparently missing only in the Iberian and Guyana Basins, the latter faunally unique for many taxa. The Pacific Ocean species *C. whitlatchi*, like *C. turnerae*, has both a very large depth distribution, extending from 2727 m to hadal depths over 7000 m, and a broad geographic range (Table II). The question arises, Why is the other abyssal *Chevroderma* species, *C. gauson*, so restricted in range? Does it have a different dispersal ability, or ecological requirements met only by the northern West European Basin?

Distribution within ocean basins. Only a few data exist on local abundances within a basin, and only for the North American and West European Basins in the Atlantic. From grabs and box cores come quantitative data on density of species as number per square meter and their rank order. From sled trawls come data on total numbers of individuals by species or higher taxa and their percent composition and rank order. For the Pacific, there are data from one ¼-m^2 box core taken in the Aleutian Trench, two ¼-m^2 box cores from the mid-Pacific, and fifteen 225 cm^2 box cores in the Panama Basin. Based on either percent of total individuals or on density per square meter, the data show that particular species of Prochaetodermatidae are numerically an important part of the fauna at certain localities in the North American Basin and eastern Pacific, but not in the West European Basin or mid-Pacific.

In the North American Basin, species diversity in the Prochaetodermatidae is low, only three species, but numbers of individuals may be very high locally for two of them: up to 400 m^{-2} for *Prochaetoderma yongei*, with 200 m^{-2} not uncommon, and up to 275 m^{-2} for *Spathoderma clenchi*, with over 100 m^{-2} not uncommon (G. T. Rowe, unpub. data). Expressed either in numerical rank order or as a percent of total fauna, *P. yongei* ranks first at depths of about 1760 m at 39°46′N, 70°37′W and constitutes more than 6 percent of the fauna [Grassle, 1977, Table 2, *Prochaetoderma* sp. (abundance recalculated here); see also Table I, this paper: OCEANUS-10 Sta. 367, 370]. In a sled trawl sample near the same location, 911 *P. yongei* formed 3.6 percent of the total fauna (Table I: ATLANTIS II-12 Sta. 73; Hessler and Sanders, 1967, Table 3). Similarly, at somewhat greater depths between 2351 and 2673 m, *S. clenchi* ranked third in species abundance and formed 5.4 percent of the fauna (Rowe *et al.*, 1982, Table 2, *Prochaetoderma* sp. A; data are lumped from samples taken at several localities).

In the West European Basin the Prochaetodermatidae are represented by at least eight species, including those from the North American Basin (Scheltema, 1985). Although the diversity is greater than in the North American Basin, the numerical abundance of two species is lower: *P. yongei*, for which quantitative data are lacking, was taken only occasionally in sled trawls in low numbers and comprised only 0.07 percent or less of all individuals; *S. clenchi* formed at the most only 0.2 percent of the fauna, and in the Bay of Biscay, greatest densities were low, 8 m^{-2}.

Chevroderma turnerae occurred in low densities throughout its range, with not more than 75 individuals taken in a single sled trawl and densities ranging from 8 m^{-2} in the North Atlantic Basin to 24 m^{-2} in the Bay of Biscay (Table I: OCEANUS-10 Sta. 353; BIOGAS XI KG-207).

Data from nonquantitative sled trawls for *P. yongei, S. clenchi*, and *C. turnerae* show similar vertical distribution patterns within the two north Atlantic basins, but, like data from quantitative gear, uneven abundances in the two slopes species. The number of sled trawl samples from all cruises are given for each basin by 500 m depth intervals in Table VII (N). (All western Atlantic samples were taken with a Sanders sled trawl; data for the West European Basin are based on samples from four types of sleds and trawls. Replicates taken on CENTOB cruises BIOGAS VI and INCAL sampled the aplacophoran fauna equally efficiently.) For each species, the number of samples in which it occurred at each depth zone was tabulated and the individuals in these samples summed (ΣX; data from Table I). From these data the average number of individuals per sample was computed for each 500 m interval ($\bar{X} = \Sigma X/N$).

All three species are most numerous in their mid-ranges, *P. yongei* and *S. clenchi* most markedly so in the North American Basin, where these two species are not only abundant, but also common, occurring in most trawl samples taken at their mid-ranges. The same species are less commonly found in the West European Basin, where they occurred in fewer than one-half the samples at all depths, and less abundant in individual samples, with 10 to one hundred-fold fewer individuals per sample. *Chevroderma turnerae* has low mean numbers of individuals per sample but is more evenly distributed across the ocean than either *P. yongei* or *S. clenchi*, occurring in about one-third of all samples in both basins.

The high variance-to-mean ratios ($s_{\bar{x}}^2/\bar{X}$) indicate a patchy distribution in both basins for all three species, as indicated also by the absolute sample sizes in Table I.

The Pacific species *C. whitlatchi* is very abundant in both the Panama Basin, where it ranges up to 178 m^{-2}, and in the Aleutian Trench, with 124 m^{-2}; at the latter location it ranked second in species abundance (Jumars and Hessler, 1976; Hessler, unpub.). In four other localities, however, total numbers taken were only 1 or 2.

Importance of Aplacophora in the deep sea fauna

The Aplacophora are usually considered an insignificant part of the faunas in which they are found. Certainly the class is small in species numbers compared to other higher taxa in the deep-sea, *i.e.*, polychaetes, bivalves, crustaceans, and nematodes. It is, however, the individuals of species that interact, and certain species of Aplacophora therefore may have an important role in a community. Although the total polychaetes or bivalves present at a particular locality may outnumber the Aplacophora, a single aplacophoran species (*e.g.*, *Prochaetoderma yongei* or *Chevroderma whitlatchi*) nonetheless may be among the most abundant species present. The Prochaetodermatidae are thought to be omnivores that feed on a wide variety of organic material, probably living and dead (Scheltema, 1981), a feeding habit that equips them well, and perhaps better than most other species, to live in an environment where food may be limiting.

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ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC MEETINGS OF THE MARINE BIOLOGICAL LABORATORY

AUGUST 20–22, 1985

Abstracts are arranged alphabetically by first author within the following categories: cell motility and cytoskeleton; ecology; fertilization and development; neurobiology and behavior; and physiology. Author and subject references will be found in the regular volume index in the December issue.

CELL MOTILITY AND CYTOSKELETON

Interactions between copepods and bioluminescent dinoflagellates: direct observations using image intensification. E. J. BUSKY, GEO. T. REYNOLDS (Princeton University), E. SWIFT, AND A. J. WALTON.

Dinoflagellate bioluminescence has been postulated to represent a defense mechanism which deters predation by nocturnal grazers such as copepods. Mechanical disturbances created by the swimming and feeding of copepods should stimulate bioluminescent flashes in dinoflagellates. These light flashes should in turn elicit startle reactions (photophobic responses) in dark adapted copepods which interrupt their feeding behavior and reduce the chances of the bioluminescent dinoflagellates being consumed. The purpose of our study was to make direct observations of the interactions between grazing copepods and bioluminescent dinoflagellates using an image intensifier. By using darkfield background illumination of an intensity much lower than that produced by the bioluminescence we were able to observe simultaneously both the behavior of the copepods and the dinoflagellate bioluminescence they stimulated. Our observations indicated that the most intense dinoflagellate bioluminescence resulted from the feeding activities of copepods and that little bioluminescence was stimulated by the disturbances created by the swimming of the copepods. Interactions between individual copepods and single dinoflagellates resulted in bright, sometimes pulsating flashes, that lasted for durations of one second or longer. This interaction usually resulted in a rapid burst of swimming speed by the copepod away from the point of interaction (a photophobic response). These observations clearly demonstrate that a single dinoflagellate is capable of interrupting the feeding behavior of a dark adapted copepod, providing further evidence that dinoflagellate bioluminescence functions as a defense against nocturnal predation by copepods.

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Association of centrioles with the marginal band in skate erythrocytes. WILLIAM D. COHEN (Hunter College, New York).

All non-mammalian vertebrate erythrocytes contain a marginal band (MB) of microtubules as part of their cytoskeletal system. There is evidence that the MB functions in cellular morphogenesis and in maintenance of mature cell shape against deformation, but little is known about its biogenesis. In certain *invertebrate* erythrocytes, centrioles act as MB organizing centers during experimentally induced MB reassembly (Nemhauser *et al.* 1983, *J. Cell Biol.* **96:** 979–989), but MB-associated centrioles have not been reported for vertebrates. Skate (*Raja erinacea*) erythrocytes have the flattened, elliptical, nucleated morphology characteristic of all non-mammalian vertebrates. Lysis with Triton X-100 under microtubule stabilizing conditions produces "cytoskeletons" in which paired dots are visible in phase contrast optics under oil immersion. As visualized in uranyl acetate-stained whole mounts or in thin sections (TEM), these are centrioles with typical 9-triplet ultrastructure and right-angle orientation. Although the centriole pairs in some cytoskeletons are distant from the MB, surveys of their distribution in preparations from different animals indicates that it is non-random, with the majority adjacent to the MB or less than 1 μm from it. Many of the centriole pairs appear to be attached to MB microtubules, or have microtubules extending from them toward the MB. In addition, in rare instances, cytoskeletons are found which have teardrop-shaped MBs, with the centriole pair at the pointed "pole" from which fibers radiate. The observations raise the possibility that centrioles function in MB biogenesis during differentiation of skate erythrocytes.

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Effects of squid axoplasmic buffers containing ATP or AMP-PNP on the stability and MAPs composition of purified brain microtubules. GEORGE M. LANGFORD (University of North Carolina at Chapel Hill), ANIBAL MELO, ERROL WILLIAMS, AND KENNETH BARBER.

Dogfish shark brain microtubules were added to squid axoplasmic buffer containing ATP or its analogue AMP-PNP. Changes in: (i) stability, as measured by polymer concentration; (ii) the high molecular weight (HMW) microtubule-associated proteins (MAPs) as determined by SDS-PAGE; (iii) the ultrastructure as revealed by negative contrast electron microscopy, were monitored. The microtubules were polymerized in 0.1 *M* MES buffer, pH 6.6, containing 0.5 m*M* $MgCl_2$, 1.0 m*M* EGTA, and 1.0 m*M* GTP. The squid axoplasmic buffer described by Allen *et al.* (1985, *J. Cell Biol.* **100:** 1736–1752) containing either; (a) 30 m*M* ATP, (b) 0.3 m*M* ATP, (c) 0 m*M* ATP, or (d) 5 m*M* AMP-PNP, was used. In the buffer containing 0.3 m*M* ATP (low ATP) or 0 m*M* ATP, a decrease in polymer concentration was observed due principally to dilution. The microtubules had normal fine structure. When the MAPs in the supernatant and in the microtubule pellet were analyzed, three major HMW bands corresponding to MAPs 1, 2, and I, and 3–5 light staining bands were seen. This pattern corresponds to that seen in MES buffer. In the buffer containing 30 m*M* ATP (high ATP), most of the microtubules depolymerized. The fine structure of the remaining microtubules was essentially normal. At high ATP, the HMW MAPs in the microtubule pellet were altered. MAP 2 appeared as a doublet and MAP I and the 3–5 light staining bands were absent. The pattern of HMW MAPs in the supernatant was very similar to that seen in the low ATP buffer. These data suggest that the upper member of the MAP 2 doublet is the phosphorylated form of this protein which is bound preferentially to the microtubules. The microtubules in the buffer containing AMP-PNP did not show the MAP 2 doublet as observed with high ATP. However, particles, 20–30 nm in diameter, were seen attached to the surface of the microtubules. The particles often served to cross-connect intersecting microtubules. The particles are thought to be the microtubule protein aggregates present in the microtubule. Attachment of the particles may involve the protein kinase.

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Endogenous and stimulated electrical and motor activity recorded directly from the giant ciliary comb plates of ctenophores. ANTHONY G. MOSS AND SIDNEY L. TAMM (Boston University Marine Program).

The spacial organization of the *Pleurobrachia* comb plate regenerative response has been studied by extracellular recording. Fine (20–50 μm tip) polished glass electrodes were attached at various positions along the 1 mm long comb plate, which was viewed by a high-speed video camera attached to a dissection microscope. This "en passant" recording technique usually immobilized and lifted away a narrow sliver of cilia, representing a few of the several thousand cells which give rise to the comb plate, while allowing the remainder to beat freely, thus indicating the motor state of the comb plate. Electrical and attendent motor activity was evoked by bipolar pulse train stimulation of the ectoderm. Clearly different compound potential waveforms were recorded at different positions along the length of the cilia. Potentials recorded immediately above the base of the comb plate (the first 50 μm) were nearly always positive and monophasic; rarely biphasic, while triphasic (action potential) waveforms were never seen. In comparison, more distally recorded waveforms consisted of both monophasic and triphasic waveforms. Thus, the synaptic signal spreads electronically along the entire length of the cilium, resulting in a positive monophasic potential in all recording locations, while the regenerative response begins a short distance from the base, and propagates distally. Propagation of the action potentials is very slow; 9 mm/s @ 8°C and 25.2 mm/s @ 22°C, giving a Q_{10} of 2.07, while the electrotonic signal spread more quickly and showed little temperature sensitivity (40.5 and 44 mm/s, respectively). Thus, (1) these cilia are not isopotential, as has been argued for the much shorter ciliate cilia; (2) the calcium channels responsible for beat modification are located virtually all along the length of the cilia, thereby suggesting (3) that the calcium sensitive machinery mediating laydown and reversal also occurs along the length of the axoneme. (4) The repolarizing conductance, seen as the positive third phase of the triphasic response, is also distributed nonrandomly over the ciliary membrane.

Extracellular recording from the comb plate has been used to explore endogenous patterns of neural and ciliary effector activity in whole animals. Pinned intact ctenophores displaying normal beat show no concomitant extracellularly recorded electrical activity in the cilia, while sudden inhibition responses are accompanied by a single spike. Rapid volleys (8–10 Hz) of action potentials occur prior to and during periods of global reversal. During unilateral reversal, seen during feeding, mouth-bending precedes volleys of synaptic and action potentials in comb plates of the reversing side, which in turn precede laydown and reversal, as seen in preparations for intracellular recording. Only synaptic responses, or no responses, are recorded from the non-reversing side. Thus, electrical responses seen via intracellular recording closely parallel endogenous electrical and mechanical activity in the intact animal.

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Calcium triggers in mitosis. Rethinking an old hypothesis. ROBERT B. SILVER (Laboratory of Molecular Biology, University of Wisconsin, Madison).

L. V. Heilbrunn first suggested calcium as a trigger for mitosis. He proposed that calcium released from the cell "cortex" activated processes leading to a "clotting of protoplasm" nuclear envelope breakdown, and completion of mitosis. A demonstration of this hypothesis has remained elusive.

Experiments tested if intracellular calcium concentration fluxes were mitosis associated and served as part of the mitosis trigger mechanism. Cells were injected with Ca^{+2} buffer to: (a) chemically clamp intracellular calcium levels and fluxes; and (b) to quantitate those calcium fluxes. Second, intracellular calcium efflux channels were blocked, to: (a) titrate the number of calcium efflux channels in the mitotic cell, and necessary for mitosis; and (b) determine if Ca^{+2} influx and efflux across intracellular membranes operated in regulating assembly and functioning of the mitotic apparatus (MA). Two cell embryos of the sand dollar, *Echinaracnius parma,* were used. One blastomere was injected; the other served as a natural. Cells were injected at times between nuclear envelope breakdown (NEB) and late anaphase. Cells were maintained in rigorously calcium free seawater.

Cells were injected with antipyrylazo III (ApIII) (30 to 100 μM) to increase calcium buffering and provide a Cd^{+2} clamp. Cells were scored for survival, timing, duration of anaphase, and MA birefringence (BR). Injections of 100 μM ApIII were toxic in mitosis and interphase. Cells lysed 120 minutes post-injection. Cells injected with 100 μM ApIII lost MA BR in mitosis. Cells injected with 50 μM ApIII arrested in their cell cycle, although abortive cleavages occurred periodically with a frequency similar to cleavage of control cells. Cells injected after NEB completed that cell cycle, but arrested in the next cycle, never initiating NEB. Thirty μM APIII injected cells cycled, and developed normal embryos synchronously with controls. This represents a calcium buffering range of 1.1 nM Ca^{+2} (permissive for injection of 30 μM ApIII) through 1.8 nM Ca^{+2} (pre-NEB inhibitory level at 50 μM ApIII).

Calcium channel blockage with 8-(diamnoethyl)octyl-3,4,5,trimethoxybenzoate (TMB-8) also prevented the cell from entering, or completing mitosis (100 μg/ml through 1000 μg/ml). The levels for mitosis delay was 300 μg/ml, when injected in interphase. Plasmolysis occurred at 500 μg/ml or greater. Cells injected in mitosis (100 through 300 μg/ml TMB-8) showed enhanced MA BR, with a concentration correlative increase in the rate of anaphase A and cleavage onset. At 300 μg/ml 400 $\times 10^6$ channels are blocked, at 50 μg/ml 700 $\times 10^6$ channels are blocked. At 1000 μg/ml (1.4 $\times 10^9$ channels blocked) MA BR was lost within 2 min, and plasmolysis occurred in 4 min. At 500 μg/ml plasmolysis occurred by 130 min. Delay of mitosis to plasmolysis is between 300 μg/ml and 500 μg/ml, a nominal difference of 280 $\times 10^6$ channels.

These data demonstrate a calcium trigger for mitosis, as a pulse of calcium just prior to NEB, intracellular (intra-membraneous) stores. This calcium pulse may be utilized to disassemble the interphase cytoskeleton, activate mitotic poles through calcium dependent phosphorylation of the mitotic poles, and thereby promote assembly of the mitotic cytoskeletion and MA. An interplay between calcium buffering molecules (calmodulin, parvalbumin, tubulin, etc.), the MA-Ca^{+2}-pump, ATP levels, and an intracellular signal, is proposed as a mitotic trigger model.

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Kinesin is found both in non-neuronal and neuronal tissues. RONALD D. VALE, BRUCE J. SCHNAPP, THOMAS S. REESE, AND MICHAEL P. SHEETZ (Marine Biological Laboratory).

We purified a novel microtubule-based force generating protein from squid optic lobes and bovine brain primarily on the basis of its high affinity for microtubules in the presence of the non-hydrolyzable ATP analogue, AMP-PNP. This protein, termed kinesin, induces microtubule movement on glass, microtubule movement in solution, latex bead movement along microtubules, and may be involved in generating movement of axoplasmic organelles along microtubules. Indeed, if kinesin supports organelle translocation in axons, it is likely to be present in a variety of cells which also transport organelles. To test this, rabbit brain, kidney, and liver were homogenized and high speed supernatants were incubated with rat microtubules (100 μg/ml) in the presence or absence of AMP-PNP (5 mM). In all tissues, AMP-PNP induced the association of the 120 kD kinesin subunit with microtubules. The amount of kinesin that cosedimented with microtubules from liver or kidney supernatants was approximately the same but was 3-5-fold less than obtained with brain. Furthermore, material released from microtubules incubated with kidney supernatant and AMP-PNP induced microtubule movement on glass and in solution. These results indicate that active kinesin is present in non-neuronal cells although it appears to be present in greater quantities in neuronal tissues.

ECOLOGY

Diapause, degrowth, and age of first reproduction in Melampus bidentatus. JAY SHIRO BALBONI-TASHIRO (Kenyon College), PATRICIA WALBORN, AND BRUCE CRISE.

Melampus bidentatus from Little Sippewisset Salt Marsh (Falmouth, Massachusetts) was described as having a three or four year life span, with two- and three-year-olds contributing to the reproductive effort during summer breeding cycles. This snail is a simultaneous hermaphrodite. Earlier studies reported three or four breeding periods each summer. These breeding cycles are coupled to spring tide inundation of the *Melampus* habitat in the upper reaches of intertidal zones in North American Atlantic salt marshes. The smallest reproductive animal was 5.8 mm.

Earlier work demonstrated age-specific tissue loss during overwintering diapause in specimens of *Melampus*. Tissue loss occurs when there is no trophic input. Some workers term such loss "degrowth" and there are indications that degrowth can be associated with seasonal or reproductive stress (outputs exceed inputs). Physiologically, degrowth represents catabolic shifts and mass flows of carbon (respiration) and nitrogen (excretion) out of a diapausing animal. Younger snails had proportionately larger physiological debits after diapause. We hypothesize that age-specific degrowth could affect age of first reproduction in *Melampus bidentatus*.

To test this hypothesis we analyzed tissues through the life-cycle, developed physiological profiles, and assessed age-specific reproduction. During May and June, 1985, we maintained cultures of *Melampus* in environmental chambers (20°C, 16h:8h::L:D) and followed reproduction in a natural population of *Melampus* in Little Sippewisset Marsh (field events correlated with those observed in laboratory populations; we did not assess age-specific reproduction in field animals). In the laboratory, we evaluated reproductive effort in the first two summer breeding periods. Three-year-olds reproduced in both periods. Two-year-old snails did not reproduce during the first breeding period (though some were larger than 5.8 mm). During the second breeding period, two-year-old snails laid eggs, but fewer than three-year-olds. We completed studies of gonad changes and organic carbon analyses of egg masses. For three-year-olds there were decreases in a size-specific gonad index; these were associated with egg-laying. The gonad index increased in two-year-olds during the first breeding but varied during the second breeding. There were no age-specific differences in the organic carbon content of egg masses, but our preliminary analyses have shown that older snails lay more eggs per mass.

Our data are consonant with the paradigm that degrowth affects the age of first reproduction.

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Size selection of Littorina littorea (*L.*) *prey by the green crab* Carcinus maenas (*L.*). ADRIAN HORACIO BOCCA (Instituto de Biologia Marina y Pesquera "Alte. Storni"–Avenida Costanera s/n–(8520) San Antonio Oeste–Rio Negro–Argentina).

Size selection of the common periwinkle, *Littorina littorea* (L.), by the green crab *Carcinus maenas* (L.) was examined in the laboratory. *C. maenas* is an active predator on a variety of intertidal animals. *L. littorea* is a common snail on intertidal shores of the northwest Atlantic coast, with different patterns of distribution, density, and growth. Snails were taken from both an exposed and a sheltered environment namely Nobska Point and Great Sippewissett Salt Marsh, respectively, on Cape Cod. Shell thickness of the snails was greater at Nobska (Tresierra pers. com.).

In the laboratory, *C. maenas* preferentially attacked *L. littorea* of intermediate size (10–12 mm). Even though the total number of snails attacked did not differ between sites, modal size attacked was slightly but significantly smaller for the snails from the sheltered than from the exposed site.

This work was supported by MBL Marine Ecology Course.

Multisensory trail detection in oceanic amphipods? CAROL E. DIEBEL (Woods Hole Oceanographic Institution), KEITH STOLZENBACH, LAURENCE P. MADIN, AND JELLE ATEMA.

Hyperiid amphipods are open ocean crustaceans which use gelatinous planktonic animals for food, shelters, and brooding space for their offspring. These associations involve varying degrees of host specificity, but few obvious correlations between gross morphology of the amphipods and the types of host they chose. Do hyperiids possess specific morphological and/or behavioral traits which determine their association with gelatinous zooplankton? Two approaches have been taken to date to analyze aspects of hyperiid behavior that may reflect their different strategies in locating hosts: (1) analysis of chemical cues using a simple Y-tube olfactometer, and (2) characterization of the trail left by gelatinous zooplankton in the water column using shadowgraph techniques and videotape recording. This work focussed on two genera of hyperiids, *Vibilia* and *Lycaea,* which differ in their degree of symbiotic association with gelatinous zooplankton—salps (Thaliacea:Salpidae). In Y-tube control tests (seawater × seawater) both *Vibilia* (8 individuals, 54 tests) and *Lycaea* (7 individuals, 46 tests) were random in their selection of a Y-arm (Chi square, $P < 0.5$). However in the stimulus tests (seawater × salp water) both species showed a significant response to the stimulus side ($P < 0.005$) [*Vibilia* (12 individuals, 131 tests) and *Lycaea* (7 individuals, 44 tests)]. Salp water in the stimulus tests is defined as water that has contained healthy salps for more than 8 hours. Salps swim forward mainly by inhaling water through the anterior aperture into the jet chamber and expelling it out the posterior aperture. Videotapes of different genera of salps (*e.g., Pegea* and *Salpa*) show a distinct trail left by this jet propulsion of the salp through the water. The trail may be characterized as a pulsed jet, which is defined as a relatively continuous jet preceeded by a vortex ring. This pulsed jet may persist in the water for as long as sixty seconds. Salp swimming speeds have been estimated at 5–10 cm/s. Thus a salp may be between 3–6 meters away from a point where the trail is still distinctive. These results show that distinctive trails (both chemically and physically) are created by salps in the ocean environment which may be used by hyperiid amphipods to help locate, at some distance, their salp hosts.

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The use of mesocosms in a eutrophic estuarine system: a case study of Green Pond. ELISABETH J. FEE, SANDRA L. AVOL (Yale University), AND MARTA A. RENZI.

The effects of increased levels of nitrogen on a eutrophic system were examined at Green Pond, an estuary located on Cape Cod off of Vineyard Sound. Parameters selected for study were fouling community settlement, phytoplankton productivity, bacterial count and P/N concentrations and ratio. The experimental design consisted of four fiberglass mesocosms (2 treatments, 2 controls) and two controls for the ambient environment. The two treatments were enriched with 15.0 μM NH_4Cl by the Marriott Bottle Technique, which allows for a constant flow rate. This was determined by calculating the flush rate of the mesocosms. Fluorescein dye was introduced and its dilution rate was measured colorimetrically over a 24 hour period. Following initial loading, nitrogen levels were monitored over one tidal exchange. Nitrogen was diluted after two hours to the ambient level, suggesting rapid uptake.

Total chlorophyll-a was measured for phytoplankton settlement on fouling plates by the method of Strickland and Parsons (1972 *A Practical Hand Book of Seawater Analysis*). No significant differences in settlement were found between treated and control mesocosms or in settlement at varying depth (two-way ANOVA, $P < .05$).

Phytoplankton productivity and bacterial numbers were analyzed by ^{14}C and epifluorescence methods respectively, and showed no changed over time.

NH_4^+ and PO_4^- concentrations were measured (Strickland and Parsons, 1972) and highly significant differences occurred over time ($P < .001$), and between the treatments, mesocosms controls and ambient controls ($P < .01$). The P:N ratio increased from 1:3 initially to a final value of 10:1, indicating increased nitrogen limitation in the system.

Daily measurements of O_2, salinity, and temperature were taken at mid-tide and remained relatively constant over time.

Results suggest rapid uptake of nitrogen, however the short study period and rapid flush rate may account for failure to detect an increase in phytoplankton or bacterial growth.

This work was made possible through support from the Marine Ecology Course, Marine Biological Laboratory.

Helpers at the nest in Crepidula fornicate *(L.)? (Gastropoda: Calyptraeidae).* PETER W. FRANK (Biology, University of Oregon, Eugene).

Pratt (1944, *Mar. Biol.* **27:** 47–49) noted that *Crepidula,* a filter-feeding, largely sessile, protandric, gregarious snail, defends itself against oyster drills *Urosalpinx cinerea.* The biting defense is evidently a major function of its radula. *Crepidula* can extend and turn its head to cover about ⅔ of the shell perimeter. Its defense is relatively unspecific and appears effective against sea stars and moon snails that co-occur with it, on subtidal rocky and depositional bottoms. Attacked by oyster drills, *Crepidula* is most vulnerable at its left rear, which the *Crepidula*'s bites can not protect and where drilling is not deterred by the snails on its top, which characteristically occupy the right side of the shell underneath. In tests on a sea table, large female *Crepidula* suffered significantly less mortality when in a group or stack than when single. *Crepidula* broods its eggs; thus superpositioned males as well as unrelated females increase egg survival. At one field site (Gansett Beach, Woods Hole) more than 90% of recently dead *Crepidula* had been drilled by *Urosalpinx.* Of 312 shells > 4 cm long, 137 had been multiply drilled, up to six times. In the laboratory, once drilling had started the victim was typically attacked by additional *Urosalpinx*. Larger slipper limpets constitute more than a meal for single oyster drills. However, given the numerous *Crepidula* at the field site, this incidence of multiple drilling is unexpected from foraging theory, unless search or handling costs were high. Either different individuals of *Crepidula* reveal their presence to oyster drills unequally, or, once attacked they become easy victims.

Facilities provided by the Marine Biological Laboratory in connection with its Marine Ecology course were essential. Provision of them is gratefully acknowledged.

Markovian models of Capitella *bioturbation*. E. D. GALLAGHER (Environmental Sciences Program, UMASS/Boston), J. P. GRASSLE, AND S. R. SPRINGER.

Four sibling species of the polychaete genus *Capitella* (spp. I, Ia, II, and IIIa) are the most abundant infauna in the polluted sediments of Boston Harbor. *Capitella* sp. Ia, the largest *Capitella* species, is the dominant in Boston's Inner Harbor. Because worm size differs drastically between seasons and among sibling species, the effects of *Capitella* on sediment stratigraphy and pore-water chemistry cannot be estimated from the abundances of the old designation *Capitella capitata*. We developed an absorbing Markov model to assess the effects of *Capitella* on sediment stratigraphy. The 27-state model has 25 transient states corresponding to the 25 1-cm sediment layers and 2 absorbing states: burial below 25 cm and radioactive decay. The probabilities of particle movement and decay in the weekly transition matrix are based on the suspected capitellid ingestion rates and the decay constant for ^{210}Pb. The sediment accumulation rate is adjusted by changing the input pulse of sediment to the 0-to-1-cm interval. "Conveyor-belt" feeders, which defecate at the surface, and "random-walk" mixers, which defecate and disperse particles at depth, can produce distinct bioturbation zones. The low population abundances of *Capitella* in Boston's Inner Harbor should not produce a well-defined bioturbation zone using ^{210}Pb. Fitzgerald (1980, M.I.T./W.H.O.I. Doctoral dissertation) found an apparent bioturbation depth of 20 cm in the Inner Harbor, which suggests bioturbation by deeper-dwelling capitellids (not yet adequately sampled), other sources of mixing, or more rapid sediment accumulation in recent years. *Capitella* sp. Ia also produces long-lived (>250 μm diameter) fecal pellets, the major type of sediment in the Inner Harbor, which may inhibit the recruitment of benthic infauna and alter the environment for chemical reactions within the sediments.

Leucine aminopeptidase specific differential growth rate in hatchery reared Mercenaria mercenaria. RONALD L. GARTHWAITE (Marine Biological Laboratory), AND DENNIS WALSH AND SUSAN TALIN (Aquacultural Research Corporation, P. O. Box AC, Dennis, Massachusetts 02638).

Literature on salinity acclimation in estuarine bivalves and on the population genetics of the enzyme leucine aminopeptidase (LAP) indicates that LAP genotype affects such fitness parameters as growth rate, fecundity, and tissue weight. These results are important to commercial aquaculture for they suggest that by choosing brood stock with the appropriate LAP genotype these fitness parameters can be increased under hatchery conditions. We conducted a study to determine if LAP genotype is correlated with growth rate in the *Mercenaria mercenaria* stocks of Aquacultural Research Corporation in Dennis, MA. The clams were the result of a hatchery spawn involving 16 females and 6 males, and were reared under conditions of 20°C, 30 ppt, pH 7.8–8.0, and constant excess food. After 42 days subsamples of large (1.10–1.65 mm diameter) and small (.40–.80 mm) clams were taken. After an additional 49 days a second subsample of large (5.3–8.0 mm) and small (<2 mm) clams was taken. LAP genotypes were determined for all four samples. No significant differences were obtained (using the G-test on alleles) between the large and small clams of the first sample. Significant differences were found between the large and small clams of the second sample ($P \ll .001$). The significant differences obtained were the result of a shift in the relative frequencies of the two common homozygote classes with one class being more common in the second large clam sample and the other class being more common in the second small clam sample. The observed differences among the samples was not due to any heterozygosity effect. Thus, within the Aquacultural Research Corporation stocks, there is a correlation between LAP genotype and growth rate, with clams possessing a certain allele growing faster, on average, than clams possessing the alternate allele.

Observations on planktonic squid (Loligo pealei, *Lesueur, 1821*) *populations in Vineyard Sound, Massachusetts*. JUNE F. HARRIGAN (Laboratory of Biophysics, IRP, NINCDS, NIH, Marine Biological Laboratory).

Limited information is available on the early life history of the Atlantic squid *Loligo pealei* in the northern part of its range. To obtain additional data, planktonic squid less than 10 mm DML (dorsal mantle length) were sampled (1983, 1984, 1985) with a 1 mm mesh plankton net in a defined study area in Vineyard Sound near the MBL. The main hatch occurred over a 20–31 day period beginning in mid-June, with peak hatch near 7/6/1983 (50.6 squid per 100 m^3 water filtered), 6/21/1984 (30.5 squid per 100 m^3), and 7/3/1985 (7.5 squid per 100 m^3). After mid-July hatching was staggered, with 0–2 squid per 100 m^3 present through September. Associated environmental factors were: water temperatures 16.8°–22.5°C, salinity 29–32 ppt, and light intensity (400–700 nm) less than 250 microEinsteins per $meter^2$ per second below 5 m. More than 90 per cent of the hatch occurred between the June and July full moons (1983, 1984) and more than 85 per cent between July-August full moons (1985). Planktonic squid were found mainly below 5 m. Samples from 0, 2, and 4 m depth contained 0–30 per cent of the squid catch at 5, 6, and 9 m. Increasing density of planktonic squid with depth corresponded to a similar increase in zooplankton density to 9 m depth. A growth rate of 0.81 ± 0.16 mm per day (or 24 mm for the first month) was estimated from the spacing of size groups separated from seven large 1983 and 1984 samples. The instantaneous growth rate for the first 106 hours post-hatching, calculated from the exponential model $y = be^{mx}$, was 0.99 per cent per hour.

Influence of patch quality on the foraging behavior of two killifish (Fundulus majalis *and* Fundulus heteroclitus). OSCAR OSVALDO IRIBARNE (Instituto de Biologia Marina y Pesquera "Alte. Storni". (8520) San Antonio Oeste. CC104. Rio Negro, Argentina).

Werme (1981, Ph.D. Dissertation, Boston University) showed that *Fundulus heteroclitus* and *Fundulus majalis* exhibited different behavior when feeding on the bottom of a tidal creek. *F. heteroclitus* selectively picked at the substrate while *F. majalis* fiercely jabbed into it. However the diet of both fish was dominated by polychaetes and small crustaceans (Baker-Dittus 1978, *Copeia* **3:** 383–389). This study examined the influence of soft bottom patch quality on the foraging behavior of these two species.

Feeding behavior was observed in a 1 $m^2 \times 22$ cm deep tank. Two 0.34 m^2 patches, one with high and one with low polychaete density (20% of the high), were placed on a sand-covered bottom. Observations employed fish averaging 30 mm long. After 4 days of isolation the fish were allowed to feed and the time spent in each patch was recorded over a 60-minute period. The fish were then preserved and gut contents analyzed.

When the high and low density assemblages were dominated by *Capitella* spp. the two species spent more time in the high density patch. This effect was more pronounced in *F. majalis. Capitella* spp. was important in both diets, but the two species showed a high preference for the largest polychaete, *Leitoscoloplos robustus.*

When the patches were dominated by *Streblospio benedicti* and *Polydora ligni,* both species chose the highest density patch although *F. heteroclitus* spent more time in the patch. Both species showed low preference for the dominant species. *Cyathura polita, Capitella* spp., *Leitoscoloplos robustus,* and *Nereis* spp. were the largest and the most important organisms in the diets.

Growth of Littorina littorea *(L.) on different diets.* DANIEL EDUARDO MARTINEZ (Instituto Argentino de Oceanografia, Avda. Alem 53, 8000 Bahia Blanca, Argentina).

The effect of different diets on the growth of the common periwinkle *Littorina littorea* was investigated in the laboratory. Snails collected from Nobska Point, Cape Cod, were divided into 6 treatment categories, each replicated 3 times, with 30 animals in each of 18 containers supplied with running seawater. All snails were of similar size (8–11 mm). Five different diets were supplied: green algae *Enteromorpha* sp. and *Ulva* sp.; *Spartina alterniflora* and *Zostera marina* derived detritus (collected from Great Sippewissett Salt Marsh, Cape Cod); rhizomes and roots of *S. alterniflora;* ground barnacles; and commercial fish chow. Snails in three containers were starved as controls. The barnacle shells had a greenish coloration, probably because of microalgae.

Growth was determined from shell lip increments after 20 days of feeding. At the beginning of the experiment the outer lip of the snails had been marked with non-toxic paint.

Snails grew significantly faster on algae than on the other food items, and faster on detritus than on barnacles. There was no significant difference between the growth of snails fed on detritus and fish chow or between the growth of snails fed on barnacles and fish chow. Neither the starved snails nor those fed a *Spartina* rhizome and root diet grew.

This work was supported by a Tinker Foundation fellowship and the MBL Marine Ecology summer course.

Interaction under laboratory conditions between two mudflat burrowing species: the amphipod Ampithoe rubricata *and the polychaete* Streblospio benedicti. SANDOR G. MULSOW (Centro Investigaciones Marinas, Universidad Austral de Chile, Chile).

The distribution of macrobenthic species is often related to the characteristics of the sediments. However, competitive interactions have been demonstrated in such communities.

The amphipod *Ampithoe rubricata* and the polychaete *Streblospio benedicti* live in similar types of sediments in Barnstable Harbor, but do not occur together at high densities. These two species showed a patchy distribution over a 6-meter transect in Barnstable Marsh as evidenced by cluster analysis (Jaccard similarity index) and ordination method (non-parametrical multidementional scaling). To test for biological interactions between *Ampithoe rubricata* and *Streblospio benedicti,* a laboratory experiment based on the survival of *S. benedicti* was performed.

The results show that the survival of *S. benedicti* was significantly lower in the presence of *A. rubricata* than when no amphipods were present ($P < 0.01$). *A. rubricata* seems to disturb the activity of *Streblospio benedicti* by its movement over and within the substrate. This is likely the cause of the laboratory results and may account for the patchy distribution of these two burrower species in the field.

The effect of arborescent bryozoans on the growth of a colonial tunicate, Botrylloides *sp.* MARTIN H. POSEY (Biology Department, University of Oregon).

In Eel Pond, Woods Hole, dense aggregations of the arborescent bryozoan *Bugula* may dominate a hard substrate patch even if they settle simultaneously with the strong competitor, *Botrylloides.* This study examined whether *Bugula* canopies can inhibit the growth, and hence overgrowth capability, of this colonial tunicate.

During July 1985, dense *Bugula* spp. canopies were allowed to form on the undersides of five 18 × 12 cm opaque polyacrylic panels in Eel Pond. In late July, one half of each plate was cleared. *Bugula* was left on the other half, but other canopy formers and most understory species were removed to create bare space beneath the bryozoans. *Botrylloides* was allowed to settle and the growth of five colonies from each treatment on each plate was monitored at 3-day intervals for 12 days. Organisms settling within 5 mm of these canopies were removed. Physical effects of the *Bugula* canopy were mimicked with artificial bryozoan colonies (made from plastic aquarium weed), including glue controls.

Although juvenile *Botrylloides* grew exponentially in the cleared areas, the mean size of colonies did not increase beneath the *Bugula* canopy. The average colony size was very significantly different between these treatments after 12 days. The artificial bryozoans did not greatly affect recruitment of *Botrylloides,* but the growth of these new recruits was retarded. Glue did not affect growth rates.

Thus, dense *Bugula* canopies may reduce the growth of an otherwise dominant competitor, *Botrylloides,* and this reduction is at least partially the result of the three-dimensional structure of these colonies. The results support the contention that indirect interactions can modify the importance of overgrowth competition.

This work was supported by the Marine Ecology course.

Between site variation in shell and body measures of Littorina littorea. ALVARO TRESIERRA-AGUILAR (Universidad Nacional de Trujillo, Peru).

Littorina littorea, one of the most common gastropods of the intertidal zone of the east coast of Massachusetts, can live under widely varying conditions. Relationships between habitat and parameters such as weight, shell thickness and shell height have been demonstrated.

The present study examined whether *Littorina littorea* from different sites differ in shell and body measurements.

Snails were obtained in July and August 1985 from high and low intertidal zones at Manomet, Nobska Point, and from the main channel and mouth of the channel in Sippewissett Salt Marsh. They were kept without food for 48 h and then killed by boiling. Their bodies were removed, dried at 60°C for 48 h, and weighed to the nearest mg. Shells were similarly dried and weighed and the shell and aperture height and width were measured to the nearest mm. Linear regression analysis was carried out for each sample using shell height as the independent variable and shell weight, shell width, and dry body weight as dependent variables. Analysis of covariance compared the regression coefficients.

At Manomet, shell shape did not differ between zones; shell thickness was greater in the low than in the high intertidal zone, as shown by greater shell height. Body weight was less for shells of a given height in the low intertidal zone.

Differences between different sites within Nobska Point and Sippewissett Salt Marsh confounded shape and weight variables, so that interpretation is not clear.

The differences at Manomet may stem from differences in (1) exposure, (2) predation intensity, (3) food supply, or (4) temperature.

I acknowledge support for this research through the MBL Marine Ecology Course.

Life history studies of Lecudina *spp. gregarines (Protozoa, Apicomplexa) parasitic in* Capitella *spp. I, Ia, and Orleans (Polychaeta).* GARY E. WAGENBACH (Department of Biology, Carleton College, Northfield, Minnesota 55057), JEFFREY S. SHIMETA, AND JUDITH P. GRASSLE.

Identification was sought of intestinal gregarines from sibling species of the polychaete genus *Capitella*. Analyses of size and general shape were conducted on mature gamonts from *Capitella* spp. I, Ia, and Orleans; these three *Capitella* spp. have the same diploid chromosome number ($2n = 20$). Syzygy, gamocyst, and oocyst development in a *Capitella* sp. I gregarine were characterized.

Infected laboratory-reared and field-collected worms were cultured at 15°C on a common source of mud. Observations of gamonts and cysts were made on live specimens using light microscopy, and developing gamocysts were examined with scanning electron microscopy. Mature gamonts were photographed and measured after dissection from the host intestine. Gamocysts attached to fecal pellets were maintained at 15°C for observation of maturation. Five morphotypes of *Lecudina* gamonts were isolated from the middle third of the gut in the three *Capitella* species. Three or four undescribed species may be represented, each specific to a host species. Two laboratory-cultured, inbred lines of *Capitella* sp. I originating from California had double infections. This suggests that gregarines in *Capitella* sp. I from California and Massachusetts belong to two different species. Syzygy between gregarines from *Capitella* sp. I occurred between pairs of mature gamonts aligned laterally. Gamocysts were attached to fecal pellets by mucus, presumably of host origin, and expelled by defecation. Oocyst development and disappearance of the gamocyst wall began within 1–3 days following release from the host. The mature, ovoid oocysts (diameter 7 μm), each containing eight sporozoites, remained agglomerated until they dissociated after approximately one week.

Ability to isolate oocysts facilitates transfer–infection experiments and life cycle studies. Such studies, along with detailed descriptions of gamonts and oocysts, will be used to characterize species and to evaluate host-specificity among *Capitella* spp. gregarines.

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Self-recognition in the sponge Microciona prolifera *(Ellis and Solander) examined by histocompatibility and cell reaggregation experiments.* SVEN ZEA (Biol. Sci., The University of Texas at Austin) AND TOM HUMPHREYS.

Noncoalescence in individuals of *Microciona prolifera* in contact was observed in the field (Waquoit Bay, Cape Cod, Massachusetts); overgrowth without apparent damage was often seen. Histocompatibility was studied in the laboratory in running seawater by parabiosis (two branches tied together), grafting (wounded surfaces of longitudinally cut halves of two branches brought into contact), and explanting (two pieces fixed near each other on glass slides and allowed to approach by outgrowth). Fusion, or molding of the tissues of two pieces, occurred in all 25 isogenic (same individual) combinations, and nonfusion, or maintenance of distinct tissue boundaries, occurred in all 28 allogenic (different individuals) combinations. All three assays gave the same results of fusion or nonfusion between any pair of individuals tested, though grafts sometimes showed necrosis and death probably due to damage suffered during cutting. A pair of adjacent, noncoalescent individuals in the field also showed nonfusion in the laboratory.

Xenogenic (different species) recognition in marine sponges occurs at the level of specific cell-cell recognition. The same phenomenon was tested for allogenic recognition in mixtures of dissociated cells unstained or stained with Hoechst 33324 dye, 2 μg/ml for 30 min, allowed to reaggregate, and followed by time lapse video recording and time series photography. Cells from different individuals consistently aggregated together. They showed no evidence of individuals recognition or segregation for at least seven days, while functional sponges were formed. Aggregation factors from different individuals were equally active in promoting reaggregation of their own cells and cells from other individuals tested. We find no evidence of self-recognition at the level of cell-cell interactions during reaggregation of *M. prolifera* dissociated cells.

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FERTILIZATION AND DEVELOPMENT

The effect of lowered pH on the cell cycle of Arbacia punctulata. Philip G. Allen Jr. (Program in Cell and Developmental Biology, Division of Medical Sciences, Harvard University).

The elevation of cytoplasmic pH at fertilization has been implicated as a controling factor in the initiation of the cell cycle in sea urchin eggs (Mazia 1974, *Proc. Nat. Acad. Sci. USA* **71**: 690–693). Previous work has described the reversible inhibition of cytokinesis in the embryos of *Arbacia punctulata* (Allen 1984, *Biol. Bull.* **167**: 516–517) using pH 6.5 sodium-free seawater (NaFSW) to lower intracellular pH (Shen and Steinhardt 1979, *Nature* **282**: 87–89). The vital DNA dye Hoechts 33342 was used to determine if the nuclear cycles continue in cleavage arrested embryos, as reported for the species *Stronglyocentrotus purpuratus* and *Lytechinus pictus* (Dubé, Schmidt, Johnson and Epel 1985, *Cell* **40**: 657–666). Incubation in pH 6.5 NaFSW led to a complete arrest of nuclear cycles at the point of treatment. This arrest was observed at all stages examined: before pronuclear fusion, at streak stage and in mitosis as late as anaphase. Inhibition of cleavage occurred in anaphase cells treated with pH 6.5 NaFSW. Cleavage arrested cells had a circumferential band of actin filaments in the area of the presumptive cleavage furrow that was detected in fixed extracted eggs by staining with rhodamine-phalloidin. No similar structures were observed in control embryos fixed at cleavage. The arrest of chromosomal cycles and cleavage was reversed with subsequent incubation in seawater or 10 m*M* NH_4Cl in NaFSW, pH 7.7. NaFSW at pH 8 did not rescue inhibited cells, suggesting that the inhibition of the cell cycle is mediated by the depression of intracellular pH. One m*M* KCN in seawater also inhibited nuclear and cleavage cycles reversibly, but inhibition was much less rapid than that observed with pH 6.5 NaFSW. Both inhibition and recovery from inhibition occured in the presence of 1 m*M* Emetine, a potent inhibitor of protein synthesis in sea urchin embryos.

Experimental analysis of polyclad turbellian development following in vitro *fertilization.* Barbara C. Boyer (Union College).

Previous deletion experiments on embryos within the egg shell membrane have shown that the embryo of the spirally cleaving polyclad turbellarian *Hoploplana inquilina* is determined early (Boyer, 1981, *Biol. Bull.* **161**: 318). However it was not possible to examine the developmental capacity of both blastomeres at the two-cell stage nor to establish the time of determination of the embryonic axes with the membrane present. *In vitro* fertilization of naked eggs permits cell separation experiments as well as deletions of specific blastomeres.

Blastomeres were separated at the two-cell stage in 38 embryos and each half developed to become a characteristic partial larva. In 12 pairs (32%) neither larva had an eye, in 8 (21%) both had one eye, and in the remaining 18 (42%), an eye was present in one and absent in the other. This variation in experimental results may be explained as a segregation of cytoplasmic eye-forming materials independently of the first cleavage plane.

Of 97 embryos in which either the A or C blastomere was deleted at the four-cell stage, 72% had one eye and 28% had two eyes. In both one-eyed and two-eyed larvae, the right ventrolateral lobe was missing in 33% and the left ventrolateral lobe in 33%. Deletion of either the B or D blastomeres in 86 embryos gave rise to 69% one-eyed and 31% two-eyed larvae; of these, 17% lacked a right ventrolateral and 21% lacked a left ventrolateral lobe. These results support the hypothesis that three of the four blastomeres are necessary for normal eye formation, and that two contain the eye precursors and the third serves as an inducer. The experiments also indicate that determination of the quadrants and therefore the embryonic axes has not occurred by the four cell stage.

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*Isolation of heparin binding growth factors from Dogfish (*Mustelus canis*) brain and retina.* Yves Courtois (Inserm-Cnrs U118, 2 rue Wilhem 75016. Paris, France) and Max Burger.

Several fish species possess a high neuronal plasticity or have continuously dividing retinal cells throughout their lifespans. To learn if these tissues contain growth factor activities similar to those found in higher vertebrates, mainly bovine EDGF, (Arruti and Courtois 1978, *Exp. Cell Res.* **17**: 283–292), we used adult dogfish retina (DFR) and brain (DFB). Twenty DFR were homogenized in an equal volume of PBS and processed as described in Courtois *et al.* (1985, *Biochimie* **67**: 265–269). DFBs were homogenized in 0.1 *M* MES, 10 m*M* EGTA, .5 m*M* $MgCl_2$, 25% glycerol, 1 m*M* ATP, .1 m*M* TLCK, .02 mg/ml leupeptin (courtesy of Dr. Langford). The homogenate was centrifuged at 100,000 × *g* and the supernatant treated as for DFR. The supernatants were dialysed against acetic acid (.1 *M*). These acidic extracts were used to stimulate the proliferation of confluent bovine epithelial lens cells (10 passages) *in vitro.* Their activities were compared with EDGF (1 ng/ml). DFB and DFR both elicited a maximum stimulation of ^{3}H-thymidine incorporation at 20 μl per ml of culture medium, similar to bovine EDGF. These activities were trypsin and heat sensitive (3 min at 100°C.) and not dialyzable. A whole preparation of DFB was loaded on 1 g of heparin-Sepharose CL-6B (Pharmacia). After washing the column extensively with 0.6 *M* NaCl, Tris pH 7.2, the material was eluted with 1.0 and 2.0 *M* NaCl. Two peaks with very high specific activities were recovered in addition to an activity which is not retained. These data strongly suggest that DFB and DFR contain at least two growth factors which can stimulate the proliferation of bovine lens epithelial cells (as well as fibroblasts AG 1523) and share many properties with EDGF I and II, two heparin-binding growth factors. The wide spectrum of activity of these factors on many cell types suggest that they may play a general role in retina development and neuronal plasticity.

We wish to thank NATO, Association Claude Bernard, and Alcon Research Institute (Fort Worth, Texas) for their financial support.

Pronuclear migration of in vitro *fertilized and activated eggs of the squid* Loligo pealei. KAREN CRAWFORD (Univ. of Illinois, Urbana).

Pronuclear migration following fertilization of the squid, *Loligo pealei,* was studied using the vital DNA stain Hoechst 33342 (Calbiochem 382065). Unfertilized, *in vitro* fertilized, or artificially activated eggs were placed in 1 μg/ml Hoechst 33342 for 20 minutes and then washed several times in filtered seawater. Nuclear activity was observed with fluorescence microscopy. Observations were made at 20–22°C. The sperm pronucleus does not move within the egg from its point of entry at the micropyle. The female pronucleus is always located at an eccentric position relative to the micropyle. One and a half hours after fertilization the female pronucleus migrates to the male pronucleus. Migration occurs in 15 minutes. The first cleavage furrow always occurs along the path of female pronuclear migration, and contains the polar bodies of meiotic maturation.

The female pronuclei of eggs artificially activated with 10 μg/ml A23187 completed meiotic maturation and migrated to a position under the micropyle, the same position as that of the sperm pronucleus in fertilized eggs. The timing of the meiotic maturation divisions and pronuclear migration were identical to that of fertilized eggs.

Female pronuclear migration was inhibited in both *in vitro* fertilized and artificially activated eggs with 5 μg/ml of either colcemid (demecolcine), vincristine sulfate (Grade 1), vinblastine sulfate salt, or nocodazol (all from Sigma).

After third cleavage, which is unequal, it is possible to determine all the axes of the developing embryo. In addition, the polar bodies are consistently found in the anterior 1st cleavage furrow of all embryos investigated and mark the previous location of the female nucleus of the unfertilized egg. This indicates that the polarity of the embryo is established and can be reliably determined before fertilization in the squid egg.

This work was supported (in part) by NIH training grant 5-T35-HD07098 to the Embryology Course, MBL, and NIH Grant 5-P41-RR01395 to Lionel F. Jaffe.

Calmodulin and germinal vesicle breakdown in Spisula *oocytes.* WILLIAM R. ECKBERG (Howard University) AND ALAN G. CARROLL.

Calmodulin has been implicated in the regulation of germinal vesicle breakdown (GVBD) in several animal groups, including starfish, vertebrates, and annelids. The involvement of calmodulin in the regulation of GVBD may be general and, therefore, basic to a fundamental intracellular mechanism regulating the process of GVBD, because starfish and vertebrates undergo GVBD in response to hormonal stimulation, whereas the annelid, *Chaetopterus,* undergoes GVBD upon contact of the oocytes with seawater.

The present investigation examines further the possible involvement of calmodulin in the regulation of GVBD by analysis of an organism in which this process is normally triggered by a third mechanism, fertilization, and to compare any calmodulin involvement in fertilization-induced GVBD with parthenogenetically initiated GVBD in the same species. We therefore treated *Spisula* oocytes with calmodulin antagonists, chlorpromazine, calmidazolium, and N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7) and the inactive W-7 analog, N-(6-aminohexyl)-1-napthalenesulfonamide (W-5), to examine the role of calmodulin in meiosis reinitiation and egg activation in this species.

Chlorpromazine and W-7 both inhibited GVBD at 50–75 μ*M,* whether triggered parthenogenetically by KCl activation or by fertilization. Calmidazolium was effective at *ca.* 3 μ*M.* W-5 was ineffective. Therefore calmodulin is involved in GVBD in this species, as well. Time course studies showed that W-7 was effective at inhibiting KCl-induced GVBD only when added within 1 min after KCl. Therefore, calmodulin participates in early events of maturation and activation in this species. However, none of the anticalmodulin drugs inhibited GVBD when induced by ionophore A23187. Studies using the calcium channel blocker verapamil showed that the period during which calcium channels are required for GVBD substantially exceeds the interval of sensitivity to W-7. Calmodulin thus acts at a stage prior to the calcium flux known to be required for GVBD.

Blastodisc formation in squid (Loligo pealei) *eggs: a possible role for microtubules.* KENNETH R. KAO (Department of Zoology, University of Toronto, Canada).

The blastodisc in fertilized squid eggs is the cytoplasmic precursor of the embryo. It develops as a yolk-free region at the animal pole; its size increases steadily between the time of fertilization and first cleavage (about 3 hours post-fertilization). To examine the role of microtubules during blastodisc formation, eggs were treated with colcemid, an inhibitor of microtubule polymerization. When eggs are exposed to 5 μM colcemid (in millipore filtered seawater, MFSW) about one hour after fertilization, they fail to develop blastodiscs. Application of colcemid after partial blastodisc formation stops its growth, and continued exposure to the drug causes disappearance of the pre-formed disc. Other microtubule polymerization inhibitors including vinblastine, vincristine, and nocodazole also inhibit blastodisc development. Lumicolcemid, an inactivated form of colcemid, does not alter normal blastodisc development when used at concentrations as high as 50 μM. The effects of colcemid appear to be specific for blastodisc formation, since eggs pulse-treated with colcemid followed by thorough washing in MFSW cleave normally, but with a reduced blastodisc. These blastodisc-reduced embryos develop blastoderms that are significantly smaller than normal.

To determine the location of the colcemid-sensitive cytoplasm, colcemid was applied locally to fertilized eggs. Microhematocrit pipettes were fire polished to reduce the opening to a diameter approximately one-third the diameter of a squid egg. Forty minutes after fertilization, eggs were aspirated halfway into the opening of the polished end with either the animal or vegetal pole facing the lumen of the pipette which was back-filled with 10 μM colcemid. When the animal region is exposed to colcemid, the blastodisc fails to form. However, when the vegetal region is exposed to colcemid, the blastodisc develops normally and cleaves into a multicellular blastoderm. When the pipette is back-filled with MFSW, the blastodisc develops normally independent of the egg's orientation, indicating that the mechanical stress imposed by the aspiration does not affect development.

These findings indicate that microtubules are necessary for the formation and maintenance of the blastodisc in squid eggs. The observation that the effect of microtubule polymerization inhibitors is localized to the animal region of the egg suggests that the animal half cytoplasm forms the blastodisc.

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Nicotinamide blocks spindle formation in activated Spisula *oocytes: meiotic chromosome movement in the absence of a spindle!* L. HERLANDS (Population Council), R. E. PALAZZO, L. I. RUBHUN, AND S. S. KOIDE.

Nicotinamide, a potent inhibitor of poly (ADP-ribosyl) synthetase, arrests cleavage in *Arbacia* embryos and inhibits polar body formation in surf clam oocytes (Sano *et al.* 1979, *Dev. Growth Differ.* **21:** 457.). KCl-activated *Spisula* oocytes, treated with 2 mM nicotinamide were stained with the vital dye Hoechst 33342. The chromosomes, when examined by fluorescent microscopy, are arrested at metaphase. When seen from a polar view, they form a tightly condensed, doughnut shaped, centrally located, cluster. This cluster later becomes more compact and migrates to the cell periphery.

Treatment of the KCl-activated oocytes, containing prominant spindles with 2 mM nicotinamide results in a loss of birefringence as viewed by polarized light microscopy. Washing eggs free of nicotinamide with seawater (3–5×) did not result in spindle reappearance within 30 min. However, treatment of these washed eggs 3 min after the final wash with 3% hexylene glycol in seawater results in the appearance of a prominant birefringent spindle. Eggs treated with 2 mM nicotinamide after KCl activation do not form birefringent spindles.

In spite of the absence of a visible mitotic apparatus, chromosomes not only form a compact doughnut-shaped cluster but also move to the cell periphery. The molecular basis of this chromosome movement is unknown.

KCl-activated *Spisula* oocytes when treated with thymidine, an inhibitor of poly (ADP-ribosyl) synthetase, tested at various concentrations up to 50 mM, all formed spindles and polar bodies.

In addition, 3-aminobenzamide, another potent poly (ADP-ribosyl) synthetase inhibitor, at concentrations of up to 4 mM, does not prevent germinal vesicle breakdown (GVBD) or polar body formation (PBF) in

KCl-activated *Spisula* oocytes. It is unlikely that the nicotinamide effect is due to an inhibition of poly (ADP-ribosyl) synthetase activity. Possible alternative explanations are that nicotinamide, via conversion to NAD, may cause a decrease in the cellular ATP pool or that nicotinamide is metabolized to nicotinic acid which may affect intracellular pH.

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"Ameboid movements" induced by inhibitors of macromolecular synthesis in clams and sand dollars. TIM HUNT AND ROBERT B. SILVER (Laboratory of Molecular Biology, University of Wisconsin, Madison).

Inhibition of protein synthesis by emetine blocks cleavage in clams, sea urchins, and sand dollars. Inhibition of DNA synthesis by aphidicolin has a similar effect, except that clam embryos undergo one (but only one) cleavage division in its presence. However, during studies of the effect of emetine on colchicine-arrested *Spisula solidissima* embryos using time-lapse video microscopy, we observed that an unfertilized oocyte that happened to lie in the field of observation suddenly shed its chorion and began to undergo strange movements after ~5 hours exposure to emetine. Subsequent tests showed that emetine (50–100 μM) caused parthenogenetic activation of some but not all batches of unfertilized oocytes. In all cases, oocytes incubated with these levels of emetine shed their chorions after 3–5 hours, and then underwent the types of movements described by Rebhun (1962 *Exp. Cell. Res.* **28**). These movements continued for several hours. They are inhibited by cytochalasin B, but not by colchicine. Fertilized or parthenogenetically activated oocytes also show such aberrant behavior after prolonged exposure to emetine.

Similar effects occur in fertilized *Lytechinus pictus* and *Echinorachnus parma* eggs after several hours incubation with aphidicolin. In these cases, movements more closely resemble cleavage divisions, but they do not go to completion. They take several hours to begin, but once started, the cells continue to subdivide their cytoplasm for a very long time. Under time lapse (64× speeded up) it looks almost as though the cells are boiling!

One explanation for the mechanism of emetine activation of clam oocytes may be that it inhibits the synthesis of unstable proteins which act as inhibitors of parthenogenesis. The synthesis of these proteins would normally be terminated by fertilization. Preliminary evidence suggests that one or two such proteins do exist.

Properties of sperm-aggregating factor purified from Spisula *oocytes.* S. S. KOIDE AND H. UENO (Rockefeller University).

A factor was isolated from *Spisula* oocytes that aggregates *Spisula* sperm and is designated as sperm aggregating factor (SAF). SAF was isolated by treating *Spisula* oocytes with a solution containing 1 M urea, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4 (Sato *et al.* 1983, *Gamete Res.* **8:** 119–127). After the extract was centrifuged, the supernatant was dialyzed against distilled water and lyophilized. The sperm extract was purified by ion exchange chromatography on DEAE-cellulose (2 × 6 cm) with a linear gradient of KCl (0–1 M). Fractions possessing SAF activity were pooled, dialyzed, and lyophilized. The active DEAE-cellulose fraction was further purified by gel filtration on Fractogel TSK HW65(F) (2 × 70 cm). The active material was eluted near the void volume.

The purified SAF showed three distinct biological effects: (1) aggregated *Spisula* sperm, but not *Arbacia* or *Chaetopterus,* (2) enhanced motility of *Spisula* sperm, and (3) induced the acrosomal reaction. SAF is capable of restoring motility to immotile sperm stored for several days at 4°C. The occurrence of the acrosomal reaction was determined by microscopic examination of sperm treated with SAF.

Since the acrosomal reaction involves Ca^{2+} influx, we investigated the dependency of sperm aggregating activity of SAF to Ca^{2+}. In the presence of EDTA (10 mM) or EGTA (10 mM) in FSW or ASW or Ca^{2+}-free ASW, SAF was inactive. When Ca^{2+} was added to these media, SAF possessed potent sperm aggregating activity.

SAF possessed the following physico-chemical properties: (1) sensitive to proteinase K treatment but resistant to trypsin, (2) resistant to heating at 60°C for 30 min, (3) does not bind to ConA-sepharose, fucose-agarose affinity resins or activated charcoal, and (4) resistant to phospholipase C. These results suggest that SAF is a high mol. wt. protein and probably not a polysacchride, nucleotide, or phospholipid. SAF interacts with a species-specific sperm membrane component and promotes Ca^{2+} uptake, resulting in an altered membrane charge distribution. The observed biological effects are the induction of sperm aggregation, increase and/or restoration of sperm motility and triggering of the acrosomal reaction.

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Cytochalasin B may inhibit the localization of morphogenetic determinants during the early development of the polyclad Hoploplana inquilina. MICHAEL A. LANDOLFA AND BARBARA C. BOYER (Union College).

An unusual cytoplasmic blebbing coincides with the formation of polar bodies in eggs of the polyclad turbellarian *Hoploplana inquilina.* A microfilament-derived mechanism may be responsible for this blebbing, which may be correlated with the localization of morphogenetic determinants during early development of these organisms.

Artificially fertilized eggs were exposed to 10, 20, and 40 μg/ml of cytochalasin-B, an effective microfilament inhibitor, to investigate its effects on blebbing and subsequent development. Eggs were exposed to the drug for 4 to 72 hours beginning 15 minutes after fertilization, and were observed continuously for 5 to 6 hours following the time of first blebbing in controls. During this period, blebbed eggs were separated from unblebbed eggs. Five to seven days later the larvae were compared to control Müller's larvae.

Over 98% of eggs in 10 and 20 μg/ml cytochalasin-B blebbed, although sometimes somewhat later than controls. The resulting larvae were normal, indicating that concentrations of cytochalasin-B equal to or lower than 20 μg/ml have a minor effect, if any, on development of *Hoploplana.* However, about 40% of eggs treated with 40 μg/ml did not bleb. These unblebbed eggs followed one of two courses of development, both abnormal: (1) some eggs divided to about the 32- to 64-cell stage, before dying. (2) The remainder appeared to develop normally to about 3 days, and then arrested as round, lobeless swimming larvae with differentiated tissues. These abnormal larvae died three days later. Eggs exposed to cytochalasin-B (40 μg/ml) which did bleb developed to normal Müller's larvae.

These data suggest a correlation between cytoplasmic blebbing, which may be involved in the localization of morphogenetic determinants, and the normal development of *Hoploplana.*

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Juvenile hormone in Crustacea. H. LAUFER,* D. W. BORST,* F. C. BAKER,[1] AND D. A. SCHOOLEY.[1] (*University of Connecticut, Storrs, and [1]the Zoecon Corporation, Palo Alto, California).

Juvenile hormone (JH) plays important roles in insect development and reproduction. We and others have shown that JH analogues, such as methoprene, also have potent effects on these same processes in crustaceans, suggesting that there may be JH-like compounds in this arthropod class. One possible source of such a crustacean JH is the mandibular organ (MO), a tissue that has been shown by others to affect reproduction in the spider crab, *Libinia emarginata.*

To test this hypothesis, we incubated MOs from several Crustacea, including *Libinia, Callinectes sapidus,* and *Homarus americanus* in Pantin's or lobster saline for 2 hours supplemented with methyl-labeled methionine. Secreted products were extracted from the culture medium and analyzed by HPLC. MOs from all three species secreted radiolabeled methyl farnesoate (MF). The chemical identification was confirmed by GC/MS. The synthetic rate for MOs from *Libinia* ranged from 3 to 38 ng/gland/h. Radiolabeled JHIII was also secreted by *Libinia* MOs, at a rate that was less than 0.1% of that observed for MF. Stereoisomer analysis of this material revealed both enantiomers, suggesting that it may have been formed by the non-specific chemical oxidation of MF. MF was also detected in hemolymph samples from *Libinia,* using a GC/MS procedure, with circulating levels of 10 to 50 ng/ml. These samples also contained small amounts of JHIII, 3-30 pg/ml. These were probably produced by the oxidation of MF during analysis.

Our data demonstrate that MF is a tissue specific, major secretory product of the crustacean MO. Since MF has JH activity in insects, and its concentration in Crustacea appears to correlate with the reproductive state of the organism, this compound may be a crustacean JH. Alternately, MF may be a prohormone, similar to ecdysone, that is converted to some other compound, such as JHIII, by peripheral tissues.

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Effect of optical isomers of gossypol on the ability of Spisula *sperm to fertilize oocytes.* S. J. SEGAL (Rockefeller Foundation), L. HERLANDS, AND M. K. SAHNI.

Recently, racemic gossypol has been resolved into highly purified (+) and (−) enantiomers. The (+) enantiomer is reported to be inactive as an antifertility agent, in the male rat, whereas the (−) stereoisomer is active. This difference in activity between the enantiomers may be due to structure-activity relationships, in addition, they may also reflect differences, in rates of gastro-intestinal absorption, in metabolic inactivation and in pharmacokinetic parameters such as biological half-life. We have compared the activities of the enantiomers *in vitro* thus eliminating many of the variables confounding the interpretation of the *in vivo* results.

A quantitative assay is used which is based on % fertilization of oocytes exposed to a standard dilution of gossypol-treated sperm. Fifty μl of pelleted sperm, prepared by centrifugation of dry sperm (1500 g, 5′) is suspended for 3 min in 1 ml of FSW containing 50 μM gossypol and pelleted by centrifugation. Controls are exposed to 0.01% ethanol v/v. These treated sperm pellets, diluted 1:20,000, are used to fertilize approx 2.5×10^4 oocytes/ml of FSW. After 80 min cultures are scored for germinal vesical breakdown and/or cleavage.

In one experiment (>1000 eggs were scored in each sample), 97% of eggs in the control were fertilized; (+/−) and (−) gossypol-treated cultures both had 25% fertilization; (+) gossypol-treated cultures had 6% fertilization. In other experiments the (+) gossypol was at least 3× more effective than (−) gossypol in reducing the ability of *Spisula* sperm to fertilize oocytes. In conclusion, (+) gossypol is more effective *in vitro* in inhibiting sperm motility and in preventing fertilization than either (−) gossypol or the racemic mixture.

MKS is a Rockefeller Foundation fellow. (+) and (−) enantiomers of gossypol were provided by Yu Yu-Wan of the Institute Materia Medica, Beijing.

An unusual mechanism for regulating the activity of ribonucleotide reductase in early development. N. STANDART, T. HUNT, AND J. RUDERMAN (Department of Anatomy, Harvard Medical School).

Fertilization of clam (*Spisula*) oocytes triggers the translational activation of three very abundant maternal mRNAs, those encoding the cyclin proteins A and B, and protein C. We have recently identified the newly synthesized protein C as the small subunit of the enzyme ribonucleotide reductase. This enzyme catalyzes the first unique step in DNA synthesis, the reduction of rNDPs to dNDPs. Since reductase is almost always composed of two subunits, a small one and a large one, both of which are required for activity, and there is no sign that a protein of the size expected for the large subunit (84K) is synthesized after fertilization, we set about to test the possibility that the large subunit already exists as protein in the unfertilized oocyte. The small subunit (protein C) was isolated by passing embryo extracts over Sepharose coupled to the monoclonal antibody YL ½ (Standart *et al.* 1985, *J. Cell Biol.* **100:** 1968) and eluting with the di-peptide glu-tyr. The small subunit, which alone has no activity, was then used to test for the large subunit in oocytes or embryos by enzyme reconstitution assays. Embryo extracts (active) were shown to have two separable components which could be combined to reconstitute activity: the YL ½ eluate (the small subunit) and the YL ½ flow-through material. Oocyte extracts (inactive) lack the small subunit, but contain a component that can be supplemented with isolated small subunit to produce reductase activity. In mammalian cells, large subunit (84K) can be isolated by binding to dATP-sepharose and elution with dATP; clam embryos extracts (active) also contain an 84K protein that binds to this matrix, the presumptive large subunit of clams. When oocyte extracts were subjected to this same type of analysis, we found that they too contained abundant amounts of this same 84K protein. This 84K oocyte protein (inactive by itself) was added to an embryo extract depleted of the 84K protein (inactive) to generate reductase activity. We conclude from these results that the oocyte contains a store of large subunit protein whereas the small subunit is stored as a precursor in the form of messenger RNA.

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Identification of mRNA binding sites in the Chaetopterus *egg cytoskeleton by* in vitro *hybridization.* BILLIE J. SWALLA (University of Iowa), JOANNA E. SPEKSNIJDER, AND WILLIAM R. JEFFERY.

Maternal mRNA molecules are localized in the cortex of *Chaetopterus* eggs. This localization is maintained by the association of mRNA with a detergent-insoluble, cortical cytoskeleton. Previous studies involving the centrifugal displacement of the cortical cytoskeleton suggested that the proper positioning of this structure or one of its components in the egg is necessary for normal development. To examine the developmental role of cytoskeletal mRNA, it is necessary to identify mRNA binding sites and to determine whether alterations in the usual mRNA-cytoskeletal interactions affect development. As an initial step, we have developed an *in vitro* system for the detection of mRNA molecules in isolated cortical cytoskeletons and have used this system to obtain preliminary information on the nature of mRNA-cytoskeletal interactions. For *in vitro* hybridization, eggs were extracted with NP-40 (Jeffery 1985, *Dev. Biol.* **110:** 217–229), and the isolated cytoskeletons were washed to remove detergent, incubated with ^{3}H-poly(U), and subsequently analyzed by scintillation counting or autoradiography after histological processing. Isolated cytoskeletons bound high levels of ^{3}H-poly(U) after *in vitro* hybridization. Thermal denaturation showed that the bound ^{3}H-poly(U) melted with a T_m of 65–70°C (0.2 M NaCl), suggesting that it formed duplexes with poly(A) in cytoskeletal mRNA. Treatment of the isolated cytoskeletons with RNase A did not release radioactive poly(U):poly(A) complexes from the cytoskeleton, implying that at least one mRNA binding site is located in the 3′ poly(A) sequence. Treatment of isolated cytoskeletons with DNase I, which depolymerizes F actin, did not affect the levels of ^{3}H-poly(U) binding obtained by *in vitro* hybridization, suggesting that actin filaments alone are not responsible for mRNA binding. We conclude that mRNA-cytoskeletal interactions are based in part on the association of poly(A) sequences with non-actin components of the cortical cytoskeleton.

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Studies on the localization of Spisula solidissima *maternally stored mRNAs and functions of a protein, Cyclin A, encoded by this message pool.* KATHERINE SWENSON (Harvard Medical School) AND JOAN RUDERMAN.

The oocytes of *Spisula solidissima* contain a pool of maternally stored mRNAs which are not utilized to make proteins until after the time of fertilization when they become rapidly loaded onto polysomes. We are interested in the mechanisms by which these mRNAs are translationally controlled and the functions of the proteins which they encode in the rapidly dividing embryo.

To gain an understanding of the mechanisms by which the maternally stored mRNAs are translationally controlled, we have localized some of them within the oocyte. Using single-stranded probes generated from cDNA clones specific for three of the maternally stored mRNAs, we hybridized sections of fixed oocytes. Each of these three mRNAs (one of which was specific for histone H3) localized not to the large germinal vesicle, which is present in the oocyte although not in the fertilized egg, but exclusively to the cytoplasm. This result contrasts with the pronuclear localization of the early variant histone mRNAs in the sea urchin egg. These results indicate that the mechanism by which translation of these mRNAs is prevented in the oocyte is not mediated *via* a simple, physical sequestering of them by the germinal vesicle from the ribosomes in the cytoplasm, but by some other, less apparent mechanism.

The Cyclin A protein which is encoded by a maternally stored mRNA was also studied. Although Cyclin A is synthesized continuously after fertilization, it is periodically and precipitously degraded before each mitotic metaphase.

Using an anti-Cyclin A antibody, we probed immunoblots containing total *Spisula* proteins obtained at different times following fertilization. From this study, we discovered that, not only does Cyclin A undergo the periodic accumulation and degradation during mitosis, which had been determined before, but it also displays the same cycling behavior during meiosis as well. We suspect that it is an important protein involved in the mechanisms which drive cell division in the early embryo. Preliminary immunofluorescence studies using the anti-Cyclin A antibody indicate that Cyclin A becomes associated with the outer cellular membrane.

Chemical nature of gossypol-macromolecule complexes. H. UENO (Rockefeller University), S. J. SEGAL, J. M. MANNING, AND S. S. KOIDE.

To understand the molecular mechanism of action of gossypol, it is important to clarify the chemical nature of gossypol interaction with macromolecules. It has been demonstrated that gossypol, a dialdehyde molecule, forms strong complexes with various amines through Schiff base linkage. However, reports claim that gossypol interacts with lactate dehydrogenase-X or erythrocytes without forming Schiff base. Because of these reports, we re-examined the chemical reaction between gossypol, a sesquinterpene, and ribonuclease, and bovine serum albumin (BSA).

BSA (0.5%) was incubated with ^{14}C-gossypol at pH 7.5, and the resulting complex precipitated with trichloroacetic acid (20%, TCA). Incorporation of radioactivity into both soluble and insoluble fractions were measured. To avoid non-specific binding of gossypol to BSA, the TCA precipitate was washed with ethanol. Over 95% of the label was recovered in the ethanol wash when BSA was incubated with gossypol alone. On treatment with 10 mM $NaBH_4$ for 10 min at pH 7.5, over 57% of the radioactivity was associated with the precipitate. The present result suggests that the principle interaction between gossypol and BSA is by Schiff base formation.

To validate this, the effect of gossypol on ribonuclease was examined. Pancreatic ribonuclease A activity (10 μM) was inhibited by gossypol (10 × molar excess) at pH 7.5, suggesting interaction of the terpene with the active site. Its kinetic pattern obeyed a pseudo-first order reaction with $t_{1/2} \sim 45$ min. The interaction of gossypol with ribonuclease was investigated by the determination of the circular dichroism spectrum (CD). A positive CD spectrum at 420 nm was observed when gossypol was incubated with ribonuclease for one hour. The present results suggest that gossypol strongly interacts with ribonuclease via a Schiff base linkage with the amino group at the active site. We propose that Lys-41 of the active site interacts with gossypol.

Cyclin gene expression in early development. JOANNE WESTENDORF (Harvard Medical School), TIM HUNT, AND JOAN RUDERMAN.

Oocytes and early embryos of the clam *Spisula solidissima* contain the same set of messenger RNA's but translate different subsets of these messages. The three most abundant messages, referred to as A, B, and C, are inactive in the oocyte and actively translated after fertilization. A and C messages have been cloned previously and a clone for B has now been isolated from a lambda gt10 cDNA library made from early embryo poly A + RNA. Identification of B clone is by gel comparison of the protein product of hybrid selected message and genuine *in vivo* synthesized protein B. The identities of proteins A, B, and C are known: A and B are cyclins and C is the small subunit of ribonucleotide reductase. Cyclins A and B are synthesized continuously during early cleavage and destroyed at each mitosis, and ribonucleotide reductase is an enzyme required for DNA synthesis. Because all three proteins are associated with the cell cycle, we asked how their expression is related to cell proliferation rates. Cell division rates were determined by counting nuclei stained with Hoechst 33342 and by the diphenylamine assay for DNA. mRNA levels were determined by Northern blot analysis. Three phases of cell proliferation were distinguished in the first 18 h of embryogenesis. From 0 to 6 h, 6 to 12 h, and 12 to 18 h, cell number and genomic DNA content doubled every 1 h, 4 h, and 6 h, respectively whereas mRNA levels for A, B, and C rose to 180%, decreased to 30%, and further decreased to less than 20% of oocyte content during the respective time periods. This finding that the quantities of A, B, and C messages were highest during fast rates of proliferation and lowest during slow rates suggests that the production of proteins A, B, and C is closely coupled to the rate of cell division throughout the first 18 h of development.

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NEUROBIOLOGY AND BEHAVIOR

Toadfish sonic motor system: I. Physiology. N. T. BENNETT, M. WEISER, R. BAKER, AND M. V. L. BENNETT (Albert Einstein College of Medicine, Bronx, New York 10461).

Toadfish generate sounds by highly synchronous contractions (up to 200/s) of the swim bladder muscles. Motoneurons innervating the sonic muscle and presynaptic axons were recorded from within the sonic motor nucleus (see p. 556, Weiser *et al.*). Antidromic stimulation of the peripheral nerve evoked antidromic spikes in motoneurons but also short latency graded depolarizations, due to electrotonic coupling between them. Orthodromic responses were evoked by stimulation in the mesencephalon. Brief trains of low amplitude evoked bursts of impulses similar in frequency and duration to the normal "vocalizations." Timing was determined with respect to the highly synchronous volleys recorded from the ventral roots intracranially. The first impulse was initiated by an EPSP arising abruptly from a nearly level baseline; each later impulse was initiated by a rapidly rising EPSP that began from a large hyperpolarizing IPSP. Polarizing currents could augment or invert the IPSP demonstrating that each EPSP was followed by an IPSP whose duration was about equal to the time between impulses. The EPSP could be decreased but not inverted consistent with electrotonic generation. The PSP patterns indicate that the discharge frequency is set in a "higher level" nucleus. Presynaptic axons whose physiological identification was confirmed by HRP injections (see p. 556) also exhibited graded depolarizations in response to sonic nerve stimulation consistent with electrotonic coupling to the motoneurons. Orthodromic activity could resemble the motoneuron discharges except that impulses occurred slightly earlier, at a time appropriate for them to generate the rapidly rising EPSPs. Some axons exhibited large subthreshold EPSPs and gradually rising EPSPs prior to the first impulse. Although these axons also showed IPSPs between impulses, the interspike potential rose more gradually. These axonal recordings appeared to be obtained from near the site which the sonic burst was initiated. Thus, the pacemaker or command nucleus for the sonic motor system may be the lateral and caudal cells of origin of the axons. Whether inhibition acts on the pacemaker process is unclear; but the termination of possible inhibitory neurons in the putative pacemaker nucleus is suggestive. In any case, inhibition of the motoneurons would increase synchronization by preventing them from firing late.

Electrosensory and visual maps in the tectum of the little skate, Raja erinacea. DAVID BODZNICK AND MARK RONAN (Dept. Biology, Wesleyan University, Middletown, Connecticut 06457).

The optic tectum of skates is divided into superficial, central, and periventricular zones. Retinal efferents terminate in the superficial zone of the contralateral tectum. The central zone receives nonvisual inputs including largely crossed second order electrosensory fibers from the medulla.

Following surgical exposure of the tectum, skates were paralyzed (tubocurare 1–5 mg/Kg, IV) and rigidly positioned in an aquarium in a dimly lighted room and ventilated with seawater (8–10°C). A stimulus device allowed positioning of visual and electrosensory stimuli at any point on an imaginary sphere of 30 cm radius centered at a point near the center of the body disk. The visual stimulus was a small light spot (560 nm, 1–2° in size); the electrosensory stimulus was a dipole E field delivered from salt bridge electrodes 3 cm apart. The resulting E field was 2 μV/cm near the fish. Multiple unit responses were recorded and quantitated using a window discriminator and spike counter.

Only visual responses could be recorded from the superficial tectal zone and composite receptive fields (RFs) were typically 20–30° in azimuth, 15–20° in elevation. Rostral to caudal visual azimuths are mapped in a rostral to caudal direction on tectum; high to low elevations are represented in a medial to lateral direction. While visual azimuth appears uniformly represented, elevation is not. A large overrepresentation exists for the 20° of visual field just above the horizon.

Electrosensory responses predominate in the central tectal zone. Electrosensory RFs are much broader than visual RFs, but their azimuths show a similar rostral to caudal progression along the rostrocaudal tectal axis. While well aligned in caudal tectum, electrosensory RF azimuths are displaced rostrally (by as much as 45°) relative to visual RFs over the rostral ⅔ of tectum. This probably results from a large overrepresentation of the rostral electrosensory field corresponding to the high density of electroreceptors located on the snout.

Effects of activation of the efferent vestibular system upon the response dynamics of the primary afferents of the horizontal semicircular canal in the toadfish, Opsanus tau. R. Boyle and S. M. Highstein (Dept. of Otolaryngology, Washington University School of Medicine, St. Louis, Missouri 63110).

Primary afferents of the labyrinthine, horizontal semicircular canal (HSC) were penetrated with glass microelectrodes in alert toadfish and their response dynamics to sinusoidal stimulation in the plane of the HSC determined. Spike trains were binned into histograms and the best sinusoid fit to the histogram using a least squares regression program. Sensitivity was defined as imps/s/deg/s of stimulation and phase by referring the peak of the response sinusoid to the stimulus sinusoid. The effects of electrical and behavioral activation of the efferent vestibular system (EVS) upon the response dynamics of afferents was determined. Records taken from the efferent branch to the posterior semicircular canal during electric pulse stimulation (0.1 ms, <0.5 mA) of the efferent vestibular nuclei (Highstein and Baker 1985, *J. Neurophysiol.*) indicated that efferent axons could follow stimulus trains up to 500 Hz. Two populations of HSC afferents were identified. The first maintained a nearly linear relationship of sensitivity and phase to angular velocity from 0.02–2 Hz. At 0.3 Hz at 30 deg/s mean discharge rate was 58.8 ± 28.1 imps/s, mean sensitivity was 1.03 ± 0.67 imps/s/deg/s, and mean phase re:velocity was 6.4 ± 10.8 deg, n = 25. No consistent effects of EVS activation upon this population of HSC afferents were noted. The second population of afferents was typically twice as sensitive to rotation as the first and led velocity by 50–90 deg across the stimulus frequency range from 0.005–5 Hz. At 0.3 Hz, ±30 deg/s mean discharge rate was 47.7 ± 25.5 imps/s, mean sensitivity was 2.46 ± 1.09 imps/s/deg/s, and mean phase re:velocity was 56.6 ± 17.2 deg, n = 14. Afferents of this latter population were consistently affected by the EVS. Two types of response were observed. In one group (n = 14) of typically silent or low spontaneous rate fibers (<13 imps/s) EVS activation raised the mean discharge rate by roughly 150% and increased the response sensitivity by 30%. In the second group (n = 13), mean discharge rate about 50 imps/s, EVS activation raised the mean discharge rate during rotation by 20% and decreased the response sensitivity by 15%. No consistent response of EVS activation upon the phase of either group was observed.

Temperature effects on physiological responsiveness of lobster chemoreceptor cells. Paola F. Borroni and Jelle Atema (Boston University Marine Program, Marine Biological Laboratory).

To examine the physiology of amino acid receptors in the taste system of *Homarus americanus,* we monitored the effects of seasonal temperature acclimation on the responsiveness of chemoreceptor cells to chemical stimuli, throughout the year (August 1984 through August 1985). Locally trapped lobsters were held at ambient seawater temperatures (−.5 to 24°C). All experiments were done at room (recording) temperature, 16–19°C year-round. Extracellular recordings were from dissected nerve bundles of excised walking legs. If no chemosensory responses to a standard stimulus were obtained from any fibers of the nerve bundle, the preparation was scored as negative; if one or more fibers responded, it was scored positive. The percent positive preparations was measured as a function of (1) season, (2) acclimation temperature, and (3) temperature shock, *i.e.*, the difference between acclimation and recording temperatures.

Acclimation temperature was the only important factor influencing physiological responsiveness of chemoreceptors: responsiveness was nil below 4°C and only 12% for temperatures ≥23°C, in comparison to values of 100 to 70% for temperatures ranging from 6 to 20°C; it decreased slowly with increasing temperatures >15°C, and fell sharply with temperatures <4°C. These results resemble those from amino acid and pyridine receptors of crayfish walking legs (Hatt 1983, *J. Comp. Physiol.* **152:** 405–409) whose function is drastically altered outside the temperature range of 5–22°C. The results suggest a correspondence between the optimal temperature range for physiological responsiveness of chemoreceptors and the greatest temperature range of the natural habitat of local lobsters (0–25°C in shallow water). While animals acclimated to <4°C hardly feed, the feeding behavior of those acclimated to ≥23°C appears to be normal. Low responsiveness of cells from the latter may be an indication of the general stress level of the whole animal which is approaching the higher limit of its temperature tolerance (28°C). This may make the preparation less resistant to the excision procedures.

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Control of light emission from cells containing endogenous Ca-activated photoprotein: roles of Ca current and gap junctions. P. BREHM, K. TAKEDA, AND K. DUNLAP (Tufts University, Department of Physiology, Boston).

The bioluminescent response of the hydrozoan coelenterate, *Obelia geniculata*, is generated by endodermal cells containing an endogenous Ca-activated photoprotein similar to aequorin. After gentle mechanical dissociation of the tissue, photocytes were identified using the fluorescence emission from the endogenous photoprotein. Isolated photocytes did not emit light following either direct electrical stimulation or high K application. However, when photocytes were associated with at least one other non-photocyte support cell, it was possible to stimulate light production by electrically driving single support cells and by high K application to the clump of cells. Removal of Ca from, or addition of Cd to the external solution blocked luminescence. Whole cell recording from photocytes, whether isolated or in clumps of cells, revealed only an outward A-type K-current, whereas support cells possessed both voltage-dependent inward Ca-current and outward K-currents. Ba could substitute for Ca as a current carrier and Cd blocked the current. Conditions which reduce Ca entry in the non-photocyte decrease light emission from neighboring photocytes, suggesting that the non-photocyte Ca-current is a critical trigger in the control of luminescence. This interaction may be mediated through gap junctions. These cells are strongly electrically coupled as shown by electrophysiological and dye coupling measurements. Application of gap junction uncouplers, octanol (250 μM), heptanol (500 μM), or Na acetate seawater (pH 6.5), reversibly blocked luminescence in response to depolarization of the non-photocytes. Octanol blocked dye coupling between non-photocyte support cells. The support cell Ca-current may trigger the passage of Ca or a Ca-dependent signal through gap junctions. Thus, in primitive excitable cell systems, gap junctions may serve in part for the control of behavioral responses.

This work was supported by a grant from the National Science Foundation.

Evidence of separate pathways for resting and excitable K-currents in squid axon. DONALD C. CHANG (Baylor College of Medicine).

The resting membrane of the squid axon has a higher permeability to K^+ ions than to Na^+ ions. However, it is not clear that such selectivity is determined by the number of Na and K channels that remain open at the resting state, or, that there may exist different kinds of "resting pathways" at the membrane which are selectively permeable to the K^+ ions. To resolve this question, we have designed an experiment to test if the K channel is the major pathway for the resting K current.

Our approach was to determine the permeability ratio (P_{Na}/P_K) of the resting membrane by measuring the effect of removal of external Na^+ on the resting potential. To maximize the sensitivity, these measurements were done in K-free artificial seawater. We then redetermined the permeability ratio after suppressing the excitable K conductance (g_K) of the squid axon by: (1) internal perfusion with 20 mM tetraethylammonium (TEA), (2) internal perfusion with 2 mM 4-aminopyridine (4-AP) plus 100 mM Cs^+, and (3) prolonged perfusion of K-free NaF internal solution. According to our voltage-clamp records, these treatments reduced the g_K by 42, 118, and 12 fold, respectively. Since the P_{Na}/P_K ratio is roughly proportional to the potential change (ΔV) associated with the removal of external Na^+, if the K channel is the major pathway of the resting K current and thus P_K is directly related to g_K, then suppression of g_K by those treatments should increase ΔV by many fold. Such a prediction was not observed in our experiment. Our measurements of 18 axons gave the following values for ΔV: -4.55 ± 1.37 (control), -5.32 ± 1.04 (TEA), 1.14 ± 0.74 (4-AP + Cs), -6.31 ± 1.23 (prolong K-free perfusion). These results indicate that the P_{Na}/P_K ratio of the resting membrane did not change significantly when most of the excitable K channels were removed or blocked. This finding suggests the existence of different pathways for the resting and excitable K currents.

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Cholinergic response in dissociated squid neurons is probably mediated by anion-selective channels. ROBERT H. CHOW (University of Pennsylvania).

In the squid stellate ganglion the neurons of the giant fiber lobe (GFL) give rise to hundreds of individual axons which fuse to form the third order giant axon. Postsynaptic potentials from GFL neurons have been described (Miledi 1967, *J. Physiol.* **192**: 379–406), but the identity of the transmitters mediating these responses is unknown.

The presence of transmitter-activated receptors in these neurons is readily studied in isolated cells which are dissociated, maintained in culture, and voltage-clamped according to procedures previously described (Bookman *et al.* 1985, *Biophys. J.* **47**: 222a). Cells were prepared one to five days before experiments. To optimize recording conditions, only cells without axonal stumps and with diameters <40 μm were selected. The membrane potential was clamped using the whole cell mode of the patch-clamp technique. Currents were recorded in response to agonist applied externally through a micropipette connected to a pressure microinjection system.

Cells were bathed in ASW, and patch pipettes contained (in m*M*) 125 KCl, 50 KF, 100 KGlutamate, 10 Hepes, and 10 EGTA. Application of 5 m*M* carbachol dissolved in ASW evoked inward currents with maximum amplitudes of 2–5 nA at a holding potential of −80 mV. These currents peaked within 350 ms and relaxed to baseline over several seconds. Similar responses were not obtained when ASW was applied. Carbachol-induced inward currents showed desensitization when carbachol was applied with intervals less than 6 s. Varying the ratio of internal to external Cl^- concentrations resulted in shifts in the reversal potential close to that predicted by the Nernst equation for Cl^- ions. Replacement of internal K^+ by N-methyl-D-glucamine and tetraethylammonium did not affect the reversal potential.

These preliminary results demonstrate the presence of cholinergic receptors on neurons of the GFL. Further experiments are in progress to elucidate the properties of these receptors and the role they play in the function of the third order fiber system.

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Optical detection of single unit activity in the isolated spinal cord of the lamprey, Petromyzon marinus. AVIS COHEN, JILL LONDON, LAWRENCE COHEN, AND HARRY ORBACH (Cornell University, Ithaca, NY).

To understand the origin of vertebrate behavior, one must monitor the simultaneous activity of large numbers of neurons. Voltage sensitive dyes and optical recording techniques have not solved the problem, however, the activity of a single vertebrate neuron has not been resolved using optical methods, although this has been achieved in invertebrate preparations.

In the experiments reported here, pieces of isolated lamprey spinal cord, 12–13 segments long, were stained with either WW376, .375 mg/ml, for 5–8 hours, or RH155, 1 mg/ml, for 5 hours. The preparation was kept cool (10°C or less) at all times. Neural activity was elicited with electrical stimulation of the cord surface. An image of the area of the cord, 1–3 segments from the stimulation site, viewed through a 32× objective, was focused on a 12 × 12 photodiode array, and absorption changes were measured as described previously.

A long (100 ms) just threshold pulse evoked a small depolarizing signal ipsilateral to the stimulation site. At the same stimulus strength, a hyperpolarizing signal was detected on the contralateral cord. The hyperpolarization followed the depolarization after a brief delay, suggesting that it was postsynaptic to the ipsilateral excitation. The response became complex with increasing current strength. A brief, large amplitude stimulus triggered a long burst of action potentials that was mainly detected on 2 diodes, each of which scanned 50 μ of the field. We think that this signal represents spike activity in an individual neuron.

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Early events in the regeneration of lateral line hair cell epithelia. JEFFREY T. CORWIN (University of Hawaii).

The initial reactions leading to regenerative replacement of amputated hair cell epithelia in the lateral line system were examined in the salamander, *Ambystoma mexicanum.* Spiedel (1947, *J. Comp. Neurol.* **87**: 29–55) briefly described lateral line reactions to amputation, reporting that sensory cilia of hair cells disappear and that dedifferentiation occurs during the first two days after amputation in the neuromasts that remain on the tail stump. These responses precede phases of cell proliferation and migration that give rise to placodes of undifferentiated cells. These invade the regenerated tail tip and form replacement neuromasts.

In this reinvestigation salamanders were anesthetized with benzocaine and a small portion of the tail tip was amputated. The reactions of the neuromasts on the tail stump near the site of the amputating cut were observed in living salamanders with light microscopy using vital dyes and DIC optics and through scanning electron microscopy in fixed specimens. These techniques demonstrated that the reported cilia disappearance is produced by active extrusion of the apical cytoplasm and the sensory cilia from the hair cells. At least ten neuromasts proximal to the cut can react in this manner starting 2 to 16 h postoperatively. All the sensory cilia of each neuromast are extruded in a single plate on top of a stalk of neuromast material that eventually extends up to 70 μm before it becomes detached from the neuromast. The extrusion of cilia is followed by a period of dramatic hair cell death that is most significant in neuromasts nearest to the cut site. Neuromasts that contained 7 to 20 hair cells before amputation contain one or two hair cells in the late phases of this cell death. Extensive cell proliferation that leads to the replacement of the lost hair cells within the neuromasts and to the accumulation of small placodal cells outside the neuromasts directly follows the period of hair cell death.

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Serial monogamy, mate choice, and pre- and post-copulatory guarding in lobsters. DIANE COWAN AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

Previous observations on the courtship behavior of lobsters, *Homarus americanus,* have shown that pre-molt females actively search for a male and cohabit in his shelter. The 1–2 week cohabitation is centered around the female molt. Mating follows molting by one half hour.

To investigate female competition we observed five female and two male lobsters (all mature: 74–81 mm CL), in a 1 × 6 m (1500 l) naturalistic aquarium for 10 weeks (June–Aug.). Although the male sizes were identical, all of the females chose the same male. While immature and young mature females in the field nearby all molted in June, the five females in this study staggered their molts throughout the summer. This molt staggering resulted in sequential cohabitation with the preferred male (serial monogamy) and implies control over the molt cycle, perhaps by male pheromones.

Surprisingly, female molt order was not correlated with dominance order. Dominance order showed linear correlation with the frequency of checking, *i.e.,* the shelter entrance of the most dominant female was investigated most frequently by the other females. We do not know the purpose of this behavior. All females frequently checked the male shelter, particularly during cohabitations. Checking by non-cohabiting premolt females peaked on days when the cohabiting female molted and mated; checking occurred least on days when the male was not cohabiting. Female checking of the male shelter was directly correlated with the intensity and frequency of male "pleopod fanning." This behavior creates a strong current through the shelter and into the environment, and may serve to advertise the male's mating status. Females rarely fan.

During the 1–2 week cohabitation the male "guards" the female. Pre-copulatory guarding may benefit females by securing the preferred male; post-copulatory shelter sharing may serve male interest in protecting his female from predation and insemination by other males.

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Circadian rhythms in the sunfish retina. ALLEN DEARRY (University of California, Berkeley), RICHARD GLABACH, BETH BURNSIDE, AND ROBERT J. BARLOW, JR.

Cone photoreceptors of the green sunfish, *Lepomis cyanellus,* exhibit a circadian rhythm in retinomotor movement. Cones elongate during subjective night and contract during subjective day in fish maintained in constant darkness (Burnside and Ackland 1984, *Invest. Ophthalmol. Vis. Sci.* **25:** 539–545). Are these morphological rearrangements reflected in changes in visual sensitivity? To investigate this, we examined the electroretinogram (ERG) of green sunfish kept in constant darkness.

Fish were adapted to the ambient light/dark cycle for at least three weeks. They were anesthetized, spinalized, and immobilized in an aquarium so that water circulated through their mouths and over their gills but their eyes remained above the water level. The ERG was recorded differentially between a conductive thread electrode contacting the cornea and a wick electrode contacting the body of the fish. Calibrated diffuse light flashes of 10 ms were delivered every 30 min to the eye while the fish was in constant darkness.

The ERG exhibited a circadian rhythm. The amplitude of the b-wave of the ERG elicited by a constant intensity stimulus of 640 nm increased at expected dusk, remained high during subjective night, and decreased at expected dawn. ERG amplitude was lower during subjective day than subjective night although a secondary increase was often seen at mid-day. Preliminary data indicate that the same response pattern is evident with stimuli of 500 nm.

The ERG intensity-response function covered a range of 4 log units, was linear at low light intensities, and saturated at high intensities. Light of higher intensity was required to elicit a given response during subjective day than subjective night. The difference between day *versus* night I-R curves indicated a 3–10-fold increase in retinal sensitivity at night.

ERG spectral sensitivity exhibited peaks at 440, 540, and 600 nm both night and day. Microspectrophotometry (MSP) revealed that rods and single cones were "green," having absorption maxima at 525 and 532 nm, respectively; double cones were "red-red," with an absorption maximum at 621 nm. "Blue" cones were not detected by MSP, but the ERG spectral sensitivity indicates their presence.

In summary, our results demonstrate that green sunfish exhibit a circadian rhythm in retinal sensitivity as well as cone retinomotor movement.

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Complex admittance of the ampullary canal of the skate Raja erinacea. H. M. FISHMAN AND A. J. KALMIJN (Marine Biological Laboratory).

The ampullae of Lorenzini enable elasmobranch fishes to detect dc and low-frequency electric fields as weak as 5 nV/cm (Kalmijn 1982, *Science* **218:** 916–918). Each ampullary organ features a skin pore, a canal, and an ampulla proper with receptor cells. The canals act as nearly ideal cables, having low core resistivity and a mural resistance of 6 Megohm · cm^2, the highest known of any biological tissue (Waltman 1966, *Acta Physiol. Scand.* **66** Suppl. 264: 1–60).

The complex admittance, $Y(j\omega)$, of a 3-cm, isolated length of canal, 1.25 mm in diameter, was measured in the frequency range 0.25–1 kHz to characterize the electrical properties of the canal wall. An isopotential was established within the length of canal by a Pt-Pt wire, passing current transmurally. The Pt wire was mounted on a voltage-sensing capillary electrode measuring the transmural potential with respect to an external reference electrode at the midpoint of the canal. The chamber was partitioned into central and end compartments by Mylar sheets with Vaseline-lined holes accommodating the canal. Cooled seawater flowed through the 3.2-mm-wide central compartment, containing a Ag-AgCl, current-measuring electrode. The canal extended from the partitions through 5-mm air gaps and passed through seawater-agar blocks in contact with Ag-AgCl guard electrodes, which were grounded to prevent current from leaking into the central compartment. The canal regions in the air gaps were rinsed with deionized-sucrose solution prior to each measurement to enhance isolation. The admittance was determined from the complex response to a 2-mV Fourier-synthesized periodic perturbation applied to the canal by a voltage clamp system (Fishman *et al.* 1983, *Biophys. J.* **43:** 293–307).

Good fits of $Y(j\omega)$ data in the ranges 0.25–100 Hz and 2.5–1 kHz were obtained to the admittance of a series circuit of a resistance and two resistance/capacitance sections. The requirement of a two-time-constant model to fit the data suggested the presence of two membranes in series with estimated resistances of 2 Megohms and 7 kohms. The 2-Megohm estimate yielded a specific resistance of 315 kohm-cm^2, which could reflect the resistance barrier of the excised canal as well as the isolation achieved in the gaps. *In vivo* measurement of the dc input resistance of 16-cm-long ampullary canals by a guarded pipet electrode, inserted into sucrose-isolated skin pores yielded resistances in excess of 250 kohms. Because this measurement gave the dc resistance of the canal and the 352-kohm ampulla (Bennett and Clusin 1978, Pp. 483–505 in *Sensory Biology of Sharks, Skates, and Rays,* Gov't Print. Off., Wash. DC), the canal resistance alone must be higher than the 63 kohm calculated for a 16-cm-long canal of 5-cm^2 surface area, based on the $Y(j\omega)$ data. Furthermore, the admittance data could be fitted without the use of a "constant phase angle" capacitance.

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Inhibition in the vestibulo-oculomotor system of the winter flounder, Pseudopleuronectes americanus. WERNER GRAF (The Rockefeller University, New York, NY 10021), ROBERT BAKER, AND HARRIET BAKER.

The thus-far-lacking conclusive demonstration of vestibular inhibition in fish questions the operational mode of the vestibulo-ocular reflex (VOR) as a reciprocal excitatory-inhibitory system throughout vertebrate phylogeny. Yet, our previous species-specific morphological data suggested putative inhibitory second-order vestibular neurons subserving the adaptation of the VOR in the postmetamorphic flatfish. We have recorded intracellularly both depolarizing and hyperpolarizing postsynaptic potentials (EPSPs and IPSPs, respectively) in antidromically identified oculomotor neurons following stimulation of the horizontal semicircular canal nerves with bipolar electrodes. In all cases, stable penetrations of somata or dendrites with large resting potentials showed that the stimulus which should have produced a hyperpolarizing IPSP induced a membrane depolarization. In the majority of motoneurons, neither depolarizing current nor Cl^- injection sufficiently changed the membrane resting potential or equilibrium potential in order to reveal a hyperpolarizing IPSP. However, in certain cells, depolarizing EPSPs and hyperpolarizing IPSPs could be demonstrated following stimulation of the appropriate vestibular nerves. Both the size and rise times of the postsynaptic potentials elicited from the prospective excitatory and inhibitory labyrinth differed, suggesting a different pattern and mode of the two respective inputs. We conclude that inhibition in the fish predominantly appears as a membrane depolarization at the soma and proximal dendrites which reduces the input resistance of the cell to produce a conductance shunt, thus short-circuiting excitation arriving at, and from, the more distal dendrites. In order to corroborate this idea, we conducted an immunohistochemical study for GABA, because it has been demonstrated to be the inhibitory transmitter of vestibulo-oculomotor neurons in higher vertebrates. The reaction product was observed in axons of the medial longitudinal fasciculus and in terminals within the oculomotor nucleus. The terminals were clearly localized on the somata of oculomotor neurons. In summary, the conceptual view of a reciprocal excitatory-inhibitory operation of the VOR throughout phylogeny—including its species-specific expression in the flounder—is substantiated by demonstrating inhibition with morphological, electrophysiological, and immunohistochemical methods.

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Ammonium "noise" improves amino acid signal detection in lobster chemoreceptive behavior. Linda S. Handrich and Jelle Atema. (Boston University Marine Program, Marine Biological Laboratory).

All sensory systems must deal with environmental noise, *i.e.*, the random background fluctuations of stimuli that contain no information. The marine environment holds rather high background levels of amino acids (10^{-8} *M*) and ammonium (10^{-6} *M*), compounds used as food signals by many marine species, including the lobster, *Homarus americanus.* We use this species, with its physiologically narrowly tuned amino acid and ammonium receptor cells, to determine how chemical signals are discriminated from the noisy background. In this experiment we raised the ammonium background concentration of natural seawater and measured its effects on the detectability of a standard, artificial food odor signal consisting of 21 amino acids and ammonium.

Physiological experiments show that ammonium receptor cells adapt completely (Borroni and Atema 1985, *Chemical Senses,* in press); thus, ammonium cells would not be stimulated until the concentration of ammonium in a mixture exceeded the background level. On this basis, one might expect decreased signal detectability due to the absence of ammonium cell contributions. However, if excitation of ammonium cells should cause central suppression of amino acid receptor cells one might expect the opposite result: adaptation of ammonium cells would release amino acid cells from suppression, causing them to respond more strongly. This should result in increased signal detectability.

Lobsters responding to a "fake" mussel—exuding the artificial food odor—showed increased responses in elevated ammonium backgrounds of 10× and 100× natural seawater levels. Signal detectability was improved in elevated ammonium noise backgrounds thus supporting the idea that amino acid cell responses were released from central inhibition. Detectability would also be improved by a reduction in "system noise," *i.e.*, the intrinsic variability of the receptor cells and CNS processing centers. Physiological experiments show that the response variance (system noise) of ammonium receptor cells is reduced in elevated ammonium backgrounds. Thus, environmental noise may play unsuspected roles in signal detection.

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The tail of Limulus *contains photoreceptors that modulate a circadian clock.* W. J. Brad Hanna, Elizabeth Pinkhasov, George H. Renninger, Ehud Kaplan, and Robert B. Barlow, Jr. (Marine Biological Laboratory).

A clock in the brain of the horseshoe crab, *Limulus polyphemus,* generates circadian rhythms in the sensitivity of the lateral compound eyes and median ocelli (Barlow *et al.* 1977, *Science* **197:** 86–89). The phase of the rhythms can be shifted by (1) illuminating the entire animal, (2) illuminating in combination the lateral eyes, median ocelli, and ventral photoreceptors, or (3) illuminating just the tail (Barlow 1983, *J. Neurosci.* **3:** 856–870; Hanna *et al.* 1985, *Invest. Ophthalmol. Vis. Sci.* **26** Suppl.: 113a).

Only the photoreceptors in the tail can alone shift the phase of the clock. Anterior photoreceptor organs must be illuminated in combinations that include at least one lateral eye plus the median ocelli or ventral photoreceptors. Illuminating the entire animal except the tail for 13 h, beginning one hour before subjective night, delays the phase by 8 h. The same illumination confined to the tail delays the phase by only 4 h.

Tail photoreceptors can influence the sensitivity of anterior photoreceptors as well as shift the phase of their circadian rhythm. For example, illuminating the tail alone for 1 h during the subjective night decreases the amplitude of the lateral eye ERG during that period. We found that tail illumination decreases lateral eye sensitivity by reducing the efferent optic nerve activity generated by the clock. Illumination of the median ocelli and one lateral eye does not reduce the sensitivity of the other lateral eye.

The rapid effect of tail photoreceptors on the clock's efferent output is not understood. Tail illumination at dusk reduces lateral eye sensitivity and delays the phase of the clock. Tail illumination at dawn also reduces lateral eye sensitivity, but advances the clock's phase. The multiple inputs to the clock may provide a key for understanding the circadian organization of the *Limulus* visual system.

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T-maze learning in the fiddler crab, Uca pugnax. DEBORAH S. KLEIN, JESSICA A. FEINMAN, AND RICHARD D. FEINMAN (State University of New York Downstate Medical Center, Brooklyn, NY 11203).

The ability of the fiddler crab, *Uca pugnax*, to learn a right-left discrimination task was studied. The animals failed to learn to choose a specified side in a standard T-maze (arms = 8 cm) even if the correct arm contained a small compartment intended as an artificial burrow. Such a narrow space, however, could serve as a reinforcer if it was immediately available in a modified T-maze with arms at a 135° angle. Correct choices were rewarded with 1–3 min in this artificial burrow; animals making incorrect choices were immediately re-started in the maze. Animals were subjected to 2 sets of 10 trials per day for 3–5 days. Of 31 crabs tested, 19 learned to turn to the specified side to the arbitrary criterion of 7 out of 10 correct. The preference was extinguished rapidly when no burrow was present. Of 11 previously trained animals, 9 were subsequently trained to choose the opposite side. The rapid extinction and the frequency of correct first choices within each block of trials suggests that, under this regimen, learning was predominantly within trials and there was little retention from day to day. That the behavior did not require visual cues was indicated by the ability of animals to perform on a schedule of alternate reinforcement, analogous to the traditional fixed ratio of 2 (FR 2) except that the first response was reinforced. Performance on the FR 2 schedule had the unusual effect of increasing the probability of appearance of the threatened posture in the animal (claw open, animal raised on walking legs). This behavior was rarely seen in animals during training or testing of controls (where there were no rewards) and the observations suggest that the behavior was evoked by the ambiguity or frustration in the schedule.

Gap junctional conductance in tunicate embryos: voltage dependence provides a plausible mechanism for formation of developmental compartments. J. KNIER AND M. V. L. BENNETT (Albert Einstein College of Medicine).

Widespread electrotonic coupling in tunicate embryos has been described previously. The discovery that the resting potential of tunicate embryonic cells could have two stable values led to the suggestion that the gap junctions might be voltage dependent, and that resting potential differences could thereby lead to compartment formation (Thompson and Knier 1983, *Dev. Biol.* **99**: 121–131). We studied coupled pairs of blastomeres of *Ciona intestinalis* and *Clavelina huntsmani* by independently voltage clamping each cell. Junctional conductance, g_j, is steeply dependent on transjunctional voltage, V_j, and is decreased symmetrically by V_js of either sign. g_j decreases more rapidly for larger V_j. For each polarity, steady state g_j is well fit by a Boltzmann relation suggestive of a constant dipole moment difference between open and closed states of the channel. Conductance is half maximal at about 12 mV and only a few percent remains at 30 mV, the approximate difference between the stable resting potentials. g_j is independent of voltage between cytoplasm and bath. The time course of the change in g_j on reversal of V_j indicates that each channel has two gates in series, and that a closed gate must open before the open gate in series with it can be acted on by Y_j. In respect to voltage dependence, gap junctions of tunicate embryos closely resemble those of amphibian embryos (Harris *et al.* 1983, *J. Neurosci.* **3**: 79–100). Junctions between isolated blastomeres were found to be permeable to Lucifer yellow (but not to sulfa-rhodamine). Lucifer yellow injected into cells near the blastopore of blastopore stage embryos often moved preferentially around the blastopore rather than axially. It remains to be shown that this apparent compartmentalization is associated with regulation of junctional conductances by differences in resting potentials. Correlation of compartmentalization with cell lineage and fate maps would suggest developmental significance.

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Elasmobranch cerebellar slices in vitro: *selective binding of potentiometric probes allows optical recording of electrical activity from different cell types.* A. KONNERTH, A. L. OBAID, AND B. M. SALZBERG (University of Pennsylvania).

We developed a brain slice preparation from the cerebellum of the skate, *Raja erinacea*, and used potentiometric probes and optical recording techniques to monitor the electrical activity simultaneously in 124 loci. We find that the fish cerebellar slice is a robust preparation with many advantages for neurophysiological studies. Handcut coronal slices (~700 μm thick) were mounted in a recording chamber containing oxygenated elasmobranch Ringer's solution and stained for 60 minutes in 0.2 mg/ml of the pyrazo-oxonol dye RH 482.

A brief stimulus (<50 μs), applied to the molecular layer by means of a teflon-coated platinum bipolar electrode, evoked large changes in extrinsic absorption that spread rapidly from the site of stimulation, but were confined to the molecular layer. These signals represent the action potential in a beam of parallel fibers. The parallel fiber action potential has a width of 3–5 ms (22–24°C) and exhibits a prominent after-hyperpolarization. The parallel fiber action potentials are blocked by TTX and prolonged by TEA. In addition, 50–100 μM Cd^{++} reversibly blocks the after-hyperpolarization, implicating a calcium-mediated potassium conductance.

When the slice was stained with the dye RH 155 (0.2 mg/ml), a close structural analogue of RH 482, the potential-dependent absorption change exhibited a second component; the parallel fiber action potential was invariably followed by a large, slow signal that reached a maximum in ~80 ms and lasted more than 500 ms. Experiments in which the slices were stained consecutively with RH 482 and then with RH 155, demonstrated that both dyes have a similar affinity for the parallel fiber membrane, but that RH 155 also stains one or more other types of cells. The slow rise time of the second component, its long duration, the sensitivity of its amplitude to the volume of the extracellular space, and the delayed recovery introduced by ouabain (1 μM), all suggest that this signal reflects transient changes in $[K]_0$ and is probably of glial origin.

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Further studies on the effect of presynaptic injection of synapsin I and Ca/calmodulin-dependent kinase II (CaM kinase II) in the squid giant synapse. R. LLINÀS, M. SUGIMORI, T. MCGUINNESS, J. GRUNER, P. GREENGARD (Dept. of Physiology & Biophysics, NYU School of Medicine, New York, New York 10016).

The actions of synapsin I and CaM kinase II on synaptic transmitter release were studied in the squid giant synapse by pressure-injecting these proteins into the presynaptic terminal. The methodology differed from our previous study (Llinàs *et al.* 1985, *PNAS* **82:** 3035–3039) as follows: (i) protein purification procedures were modified, yielding an increased affinity of synapsin I (gift of M. Bahler and R. Jähn) for synaptic vesicles, and a higher purity of CaM kinase II. (ii) The proteins and TEA/Cs were injected into the preterminal using separate electrodes; TEA/Cs injection improved blockage of K current, increasing the accuracy of inward Ca current (ICa) measurement. (iii) The stellate ganglion was bathed with 0.2 mM Ca artificial seawater (ASW), while 10 mM Ca ASW was continually superfused directly over the presynaptic digit under study. Na and K currents were blocked by addition of 5–10 μM TTX and 5–20 mM 3AP.

Presynaptic injections of synapsin I reduced transmitter release as determined by the amplitude of the postsynaptic potential evoked by command presynaptic depolarizing pulses, confirming our previous findings. This reduction occurred without a significant modification of either the amplitude or time course of presynaptic ICa. Moreover, a reversal of the depression of transmitter release was observed between 25 and 75 min, such that by 75 min transmission returned nearly to control levels.

Presynaptic injections of CaM kinase II were made during simultaneous pre- and postsynaptic voltage clamping (Llinàs and Sugimori 1978, *Biol. Bull.* **155:** 454) using several levels of presynaptic voltage pulses. In agreement with the previous study, an increase in transmitter release was observed following kinase injection. Transmitter release increased up to six fold at 25 min after injection, while presynaptic ICa as a function of command pulse amplitude was unchanged.

The present results support the hypothesis recently proposed that CaM kinase II, by regulating phosphorylation of synapsin I, modulates transmitter availability in the squid giant synapse.

Loss of potassium channel activity in the absence of external calcium ions. J. LOPEZ-BARNEO AND C. M. ARMSTRONG (Marine Biological Laboratory).

We have tested the hypothesis that Ca ions are a necessary factor in the gating of K channels. Specifically, we wanted to know if it is obligatory that K channels be occupied by a Ca ion when they close. We isolated neurons from the giant fiber lobe of the squid stellate ganglion, and studied them with the whole-cell variant of the patch clamp technique. These cells are convenient for K channel studies since they have primarily K channels in their membrane. In most experiments the internal solution contained, in mM: 550 K, 50 Cl, 25 F, and 475 glutamate. On removing Ca from the external solution (going from 10 Ca, 50 Mg, 435 Na to 515 Na), time-dependent K channel current decreases concurrently with an increase of current through "leak" channels that appear to be always open and have a reversal potential near zero mV. Eventually a square step of current is apparent. The current is inward at −80 mV and outward at +60 mV. On returning Ca (or Ca and Mg) to the external solution, the "leak" current disappears almost immediately and K channel activity reappears somewhat more slowly, in some cases recovering almost completely. The "leak" channels conduct Tris and NMG as well as Na ions. From the time course, we believe that on removal of Ca, K channels lose their gating properties and selectivity and are converted into non selective "leak" channels that are always open. Mg is not effective in preventing this K channel transformation. Ba, a potent K channel blocker, occupies and blocks the channels and prevents their transformation. Our observations support the idea that K channels must contain a Ca ion when they close and that they drastically change properties when Ca is not present.

Predicting the entire spectral absorbance curve of any visual pigment from measurements on two template pigments. EDWARD F. MAC NICHOL, JR. (Marine Biological Laboratory).

Visual pigments in mixed retinas are hard to characterize accurately because single pigments present in small amounts cannot be purified. Microspectrophotometry permits pigment sampling in a single receptor. Accurate measurements of bleachable pigments in volumes as small as one cubic micrometer are often necessary and very difficult. Noise and shortwave-absorbing contaminants distort the broad, flat, unsymmetrical peak so that it is often impossible to obtain an accurate estimate of the wavelength or frequency (F_{max}) of peak absorbance. In contrast, the rise of the low-frequency (longwave) side of the peak is steep, nearly linear, and uncontaminated by other substances. A regression line can be fitted to this region to interpolate the frequency ($F_{.5}$) of half-maximal absorbance.

Our best experimental data and those of others appear to fit the scaling law proposed by Mansfield, according to which the frequency (F) of any point having a fraction (A/A_{max}) of maximum absorption is a fixed fraction of F_{max} for all visual pigments of a given series (retinal or dehydroretinal). The curve relating A/A_{max} to F/F_{max} is invariant for each series. Therefore, $F_{.5}$ is a fixed fraction of F_{max} and can be employed to predict it much more accurately than it can be measured. Using the table of absorbances of frog rhodopsin that Dartnall used to construct his famous nomogram, F_{max} is found to be $1.0924 \times F_{.5}$ for retinal pigments. From our data and those of Munz and Schwanzara, $F_{max} = 1.110 \times F_{.5}$ for dehydroretinal pigments. The "goodness" of experimental data can be estimated by the coefficient of correlation of the regression line with the data points, and the product of $F_{.5}$ and the slope (about 8.5 for retinal pigments).

I thank Drs. J. S. Levine, R. J. W. Mansfield, S. Oleszko-Szuts, L. E. Lipetz, and Ms. B. A. Collins for their collaboration, and NIH Grant EY05046 for support.

Dynamic properties of skate horizontal cells. KEN-ICHI NAKA (National Institute for Basic Biology, Okazaki, Japan), RICHARD L. CHAPPELL, MASANORI SAKURANAGA, AND HARRIS RIPPS.

The skate (*Raja erinacea* and *R. oscellata*) retina is reported to contain only rods, the photoreceptors that subserve nocturnal vision in vertebrates. Nevertheless, Dowling and Ripps (1971, *J. Gen. Physiol.* **58:** 163–189) showed that skate retinal neurons have the remarkable capability of responding to incremental flashes superimposed on backgrounds covering a large range (>5 log units) of ambient illumination, *i.e.*, extending well above the level at which rods saturate in mixed (cone/rod) retinas. To examine further the unusual properties of the skate visual system, we have analyzed the responses of horizontal cells to step, sinusoidal, and white-noise stimuli at various mean retinal illuminances.

We find that following exposure to ambient intensities bright enough to block completely responses to both incremental and decremental stimuli, the horizontal cell undergoes a slow recovery process during which there is a marked non-linearity in its response properties. The cell responds first (within 2–3 min) to decrements in intensity, and only later (after >10 min) becomes responsive to incremental stimuli. However, after fully adapting to even the brightest mean levels of illuminance, the responses to intensity modulation are nearly linear over a broad range of modulation depths.

In addition, response amplitudes for a large range of ambient illumination indicate that the Weber-Fechner relationship describes the cell's differential threshold to random (incremental and decremental) stimuli. Departure from Weber-Fechner is seen only at the lowest mean illuminances.

Another surprising finding concerns the phenomenon of surround enhancement, observed previously in catfish and turtle horizontal cells. Although exposure to a steady annulus of light induced response compression and a pronounced sensitivity loss in response to incremental light flashes, it produced a faster, shorter-latency response and a significant increase in the cell's sensitivity to a central spot when tested with sinusoidal or white-noise stimuli.

Our results suggest that skate horizontal cells (and perhaps all of its retinal neurons) function optimally in an environment in which ambient illumination changes slowly, thus allowing ample time for adaptation; once adapted, the cells respond rapidly and linearly to any transient fluctuations in the mean ambient illumination.

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Pressure injection of calcium into Limulus *photoreceptors activates the light-sensitive sodium conductance.* RICHARD PAYNE, D. WESLEY CORSON, AND ALAN FEIN (Marine Biological Laboratory).

Brief (<500 ms) pressure-injection of 1–10 pl of 2 m*M* calcium aspartate or calcium chloride (dissolved in 100 m*M* potassium aspartate and 10 m*M* HEPES) into the light-sensitive region of *Limulus* ventral photoreceptors results in a rapid, 20–40 mV depolarization that lasts approximately 2 s. The depolarization closely follows the rise in intracellular free calcium, as indicated by aequorin luminescence. The depolarization is followed by reversible desensitization of responses to light. Similar, single injections of calcium into the light-insensitive region of the receptor are essentially without effect, even though aequorin luminescence indicated a large, rapid rise in intracellular free calcium. Prior injection of calcium buffer abolishes all the effects of calcium injection.

Injection of 2 m*M* strontium into the light-sensitive region also depolarizes and desensitizes the photoreceptor. Injection of 2 m*M* barium did not depolarize the photoreceptor, but it desensitized subsequent responses to light. Injection of 2 m*M* magnesium had no effect. The magnitudes of the depolarizations induced by the divalent ions were in the series: Ca > Sr ≫ Ba, Mg.

The depolarization caused by injection of calcium arises from the activation of an inward current with a reversal potential (between +10 and +20 mV) and rectification characteristics that are similar to those of the light-activated conductance. The reversal potentials of the light- and calcium-activated currents shift similarly by 23–35 mV to more negative potentials when ¾ of the extracellular sodium is replaced by sucrose, but are not affected by a similar replacement of sodium by lithium. Both conductances therefore appear to be similarly permeable to sodium and lithium. Light-adaptation, or prior injection of calcium, diminish the calcium-activated current much less than they diminish the light-activated current. We suggest that calcium activates a late stage in the cascade of visual transduction, so as to open the light-sensitive channels.

The isolation and characterization of cilia from the larvae of marine bryozoans. CHRISTOPHER G. REED (Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire).

The larvae of marine bryozoans are covered by a mosaic of motile and sensory cilia that constitute discrete locomotory, mechanosensory, chemosensory, and photosensory organs. Several approaches have been employed to determine if the membranes of various types of cilia exhibit differences in protein composition and organization indicative of their respective functions. Cilia from the larvae (*Bugala simplex, B. turrita,* and *B. stolonifera*) from Eel Pond have been isolated by cold (5°C) and/or hypertonic (30 gm NaCl/l seawater) shock for subsequent characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The cilia of all three species exhibit the following hierarchy of increasing tolerance to deciliation by hypertonic shock: (1) motile cilia of the locomotory organ, (2) mechanosensory cilia of the apical disc, (3) chemosensory cilia of the pyriform organ, and (4) photosensory cilia of the eyespots. Alternatively the motile cilia alone can be preferentially removed by agitation of larvae chilled to 5°C. The sensitivities of the various types of cilia to cold and osmotic shock thus appear to be organ-specific, but not species-specific. The membranes of isolated cilia were subsequently solubilized in 0.25% Nonidet P-40 and the axoneme and solubilized membrane fractions were analyzed by 5–15% gradient SDS-PAGE. Analyses by densitometry reveal that membrane fractions from motile cilia of *B. simplex* isolated by cold shock exhibit an enriched low molecular weight band (31.5 K) over membrane fractions from cilia subsequently isolated from the same larvae by hypertonic shock. No other appreciable differences were noted between the protein profiles of cilia isolated by cold and hypertonic shock. Because the fraction of cilia isolated sequentially by hypertonic shock following cold treatment contains both motile and mechanosensory cilia, the enriched low molecular weight band found in preparations of cilia isolated by cold shock alone may represent a membrane protein specific to the motile cilia of the locomotory organ.

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Toadfish sonic motor system: II. Morphology. M. WEISER, N. T. BENNETT, M. V. L. BENNETT, AND R. BAKER. (New York University Medical Center, New York, New York 10016).

The toadfish, *Opsanus tau,* produces sound with two symmetrically placed muscles wrapped around either side of the swimbladder. Retrograde labeling from HRP injections of the muscles showed a midline nucleus in the center of the rostral spinal cord with each half consisting of about 800 densely packed motoneurons. Individual dendritic trees crossed the midline and extended beyond the lateral and caudal borders. Intracellular injections of HRP into 15 sonic motoneurons showed each cell to have 4 or 5 thick proximal dendrites with secondary branches extending throughout the nucleus. Distal dendritic arborization of motoneurons, especially those located caudally, ended with a peculiar curly-cue in cellular areas lateral to the sonic motor nuclei. Most frequently axons arose from somatic initial segments. However, origin from proximal dendrites also was observed. Axon collaterals were not found within the spinal cord. We conclude the extensive size and apposition of the dendritic trees may, in part, underlie the close electrotonic coupling and synchronization during the sonic motor discharge. Axons of 11 presynaptic neurons were physiologically identified (see p. 546, Bennett *et al.*) and intracellularly injected with HRP. Six of these cells were located lateral to, and three caudal to, the sonic motor nucleus. Somas were fusiform with a large proximal dendrite immediately giving rise to 4–6 branches extending laterally and caudally. Each cell terminated bilaterally in the sonic motor nucleus and throughout the area containing the cells of origin. The somas and dendritic trees of two smaller stellate-like cells were located ventrally within the sonic motor nucleus. Terminal arborization was largely within the nucleus although collaterals were seen in the above lateral cellular areas. These morphological data suggest that the prenuclear signals generating the sonic motor discharge originate from neurons in specialized areas lateral and caudal to the sonic motor nucleus.

Light stimulates the rapid formation of inositol-trisphosphate in squid retinas. S. F. WOOD, M. S. REID, E. Z. SZUTS, AND A. FEIN (Marine Biological Laboratory).

For invertebrate photoreceptors, light's effect on excitation and adaptation can be mimicked by the intracellular injection of inositol-trisphosphate ($InsP_3$) in the dark (Fein *et al.* 1984, *Nature* **311**: 157; Brown *et al.* 1984, *Nature* **311**: 159). To test whether $InsP_3$ is a natural intermediate of transduction, the levels of $InsP_3$ were measured in squid retinas within seconds after a light-flash, over the period during which excitation and adaptation occurs. Squid retinas contain numerous receptors ($\sim 10^7$). These are the only neurons present and comprise most of the tissue volume. Retinal slices were incubated for 3 hours at 10°C in oxygenated artificial seawater containing (^3H)inositol (100 μCi; total [inositol] = 7 μM). Recordings of electroretinograms confirmed that retinas remained light sensitive after incubation. Preincubated retinas were stimulated with a flash (~ 1 ms) capable of eliciting saturating receptor responses and the biochemical reactions were quenched by plunging the tissues into Freon 22 ($\sim -150°C$) and thawing them in 15% trichloroacetic acid. This method quenches the reactions in <0.8 s. Unstimulated retinas from the same animal served as controls in each experiment. Extracted inositol phosphates were separated by ion-exchange chromatography. To correct for variability in uptake, metabolism, and extraction, the amount of (^3H)$InsP_3$ in each tissue was expressed as % of total (^3H)inositol phosphates. In control retinas, (^3H)$InsP_3$ was about 5% of the total and varied less than 14% (S.D.) within any experiment. Light increased $InsP_3$ levels. The light/dark ratio for (^3H)$InsP_3$ was: 2.36 ± 1.07 (mean ± S.D.; $n = 4$) at 0.5 s, 2.42 ± 0.79 ($n = 12$) at 3 s, and 1.12 ± 0.55 ($n = 2$) at 2 min after the flash. $InsP_3$ was formed by the hydrolysis of phosphatidylinositol-bisphosphate rather than by the phosphorylation of inositol-bisphosphate ($InsP_2$), because the light/dark ratio for (^3H)$InsP_2$ was 1.09 ± 0.23 ($n = 12$) 3 s after flash. In conclusion, light stimulated formation of $InsP_3$ is sufficiently rapid for $InsP_3$ to mediate adaptation and possibly excitation in squid photoreceptors.

A first try at optical recording from Aplysia *abdominal ganglion during habituation and sensitization of the gill-withdrawal reflex.* DEJAN ZECEVIC, JILL A. LONDON, AND LAWRENCE B. COHEN (Yale University School of Medicine).

We examined if optical methods, with the capability of monitoring activity from many neurons simultaneously, could provide additional information about the origins of plasticity in the gill-withdrawal reflex. An isolated siphon preparation (Kupferman *et al.* 1974, *J. Neurophysiol.*), with the abdominal ganglion connected by nerves to the siphon and gill, was used.

The ganglion was stained with a .5 mg/ml solution of voltage-sensitive oxanol dye NK3041 (RH155) for 30 minutes. An enlarged image of the ganglion was formed on a 124 element photodiode array. The output of each detector in the array was amplified, digitized, and recorded every 1 ms. Gill movement was recorded on video tape. We did not detect pharmacological effects or photodynamic damage. Activity in the abdominal ganglion was observed during gill-withdrawal reflex evoked by mechanical stimulation of the siphon. The number of cells whose activity was recorded optically ranged between 8 and 41 under different experimental paradigms.

In the best analyzed experimental series, in three control stimulations, an average of 16 different neurons were found active during the gill withdrawal. Most of this activity was correlated with the gill movement. The gill-withdrawal reflex was then habituated by repeated mechanical stimulation of the siphon (15 stimulations, 1 stimulation/min). In two optical recordings during stimulation of the habituated preparation, an average of 10 neurons were active. Furthermore, those neurons that were active made fewer action potentials. The gill contraction was reduced to 25% of control. After a recovery period of 20 min, 16 neurons were found active and gill contraction recovered to 90% of the control. The reflex was subsequently sensitized by electrical stimulation (6/s) of both connectives for 5 s. In the subsequent trial, we found that 41 neurons were active.

It appears that plasticity of the reflex is reflected in both number of active neurons and the frequency of firing of active cells.

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Choline acetyltransferase immunohistochemical evidence for cholinergic projections from the nuclei isthmi and reticularis mesencephali to the optic tectum in the goldfish Carassius auratus. STEVEN J. ZOTTOLI (Williams College), KENNETH J. RHODES, AND ELLIOTT J. MUFSON.

The fish optic tectum receives input from the retina and most major brainstem areas including the nuclei isthmi and reticularis mesencephali. Although various neurobiological characteristics of the optic tectum, and the nuclei isthmi and reticularis mesencephali have been studied in the goldfish, little is known about the putative transmitters of these neural systems.

We processed goldfish central nervous tissue for the immunohistochemical detection of choline acetyltransferase (ChAT), the enzyme responsible for synthesizing acetylcholine, using the monoclonal antibody AB8 and the peroxidase-antiperoxidase procedure. No staining was seen in control sections in which either AB8 was omitted or replaced by an irrelevant IgG in the immunohistochemical procedure. Bands of ChAT immunoreactivity were seen in various layers of the tectum; the heaviest staining occurred in those lamina known to receive retinal input. Numerous ChAT positive pyriform perikarya were found in the periventricular gray zone and their processes were traced to the more superficial tectal layers. ChAT containing neurons were also observed in nuclei isthmi and reticularis mesencephali and their immunoreactive fibers were seen to enter the upper layers of the optic tectum forming numerous varicosities.

Since virtually no ChAT-like immunoreactive fibers were observed in the optic tract, we suggest that the ChAT positive neurons located within nuclei isthmi and reticularis mesencephali provide cholinergic input to the optic tectum in the goldfish.

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PHYSIOLOGY

Stimulus-response coupling in Microciona prolifera: *phorbol esters and calcium ionophores act synergistically to aggregate cells in the presence and absence of extracellular calcium.* LENORE AZAROFF, SARAH DAVIDSON, PHILIP DUNHAM, AND GERALD WEISSMANN (Marine Biological Laboratory).

Movements of calcium play a critical role in the aggregation of dissociated marine sponge cells: Ca-depleted cells (in 2.5 mM EDTA) aggregate promptly upon addition of Ca (5–20 mM) and Ca ionophores (A23187, ionomycin; 5–20 μM). Sponge cell aggregation resembles stimulus-response coupling of higher organisms in which activation of protein kinase C and movements of intracellular Ca (Ca_i) may provide twin signals (Nishizuka 1984, *Nature* **308**: 693–698). We now report that activators of protein kinase C (phorbol esters) and ionomycin act synergistically to aggregate sponge cells: significant increments in light transmission of stirred cells ($P < 0.001$ *vs.* either agonist alone, n = 58). Surprisingly—since extracellular Ca (Ca_o) is required for integrity of the species-specific aggregation factor (MAF)—phorbol esters and ionomycin provoked aggregation in the absence of Ca_o (2.5–20 mM EDTA), although 10 mM Ca in excess of EDTA enhanced rate and extent. The order of activity of phorbol esters (50 nM–5 μM) and related compounds was the same as their effect on protein kinase C (phorbol myristate acetate, phorbol dibutyrate > phorbol diacetate ≫ phorbol, 4 α phorbol). 1-oleyl, 2-acetyl glycerol (OAG, 5–50 μM), a synthetic protein kinase C activator, also showed synergy with ionomycin. Phorbol esters and OAG acted in synergy with ionomycin to provoke increments in the loss rate of fluorescence of cells prelabeled with chlorotetracyline (presumably: mobilization of membrane Ca). Urushiol, the active lipid from poison ivy, but not pentadecylcatechol (its inert analogue), also showed synergy with ionomycin. Synergistic aggregation could be inhibited by indomethacin (100 μM) and pertussis toxin (20 mg/ml). The data not only confirm that marine sponge cell aggregation follows the general sequence of stimulus-response coupling in the cells of higher organisms, but support—in this primitive system—the twin signal hypothesis that mobilization of Ca_i and activation of protein kinase C mediate stimulus-response coupling in parallel.

Acoustic signals from contracting frog muscle. DANIEL T. BARRY (University of Michigan).

Contracting skeletal muscles emit continuous, low frequency sounds. The amplitude of these sounds correlates well with force production in humans and clinical uses of these sounds are emerging ("acoustic myography") (Barry *et al.* 1985, *Muscle and Nerve* **8:** 189–194). The experiments described here investigate the mechanism of sound production.

Frogs (*Rana pipiens*) were anesthetized and gastrocnemius muscles were dissected with the nerve intact. The muscle was mounted between a Grass FT03 force transducer and a fixed post in a chamber filled with frog Ringer's. Acoustic signals were recorded with a Grass 1010 microphone. Compound muscle action potentials (CMAPs) were recorded extracellularly; muscle stimulation was via the peripheral nerve.

Simultaneous records of force, acoustic, and CMAP signals were obtained for maximal twitches over a temperature range of 7.5–26°C at lengths from slack to 14 mm of stretch. The initial acoustic deflection always occurred after the initiation of the CMAP (difference of about 9 ms at 8.5°C and 4 ms at 26°C) but well before the initiation of force or displacement (difference of about 20 ms at 8.5°C and 5 ms at 26°C). The peak-to-peak acoustic amplitude increases with increasing temperature with a Q_{10} of approximately 2.5. Amplitude also increases with length up to approximately 3 mm past slack and then decreases. This pattern is similar to a plot of force *versus* length but is shifted towards shorter lengths.

These results indicate that the acoustic signal cannot be explained by a simple thickening of fibers secondary to shortening because it begins during the period of excitation-contraction coupling. A volume change occurs during this time but it is too small (10^{-5} ml/g) to generate the acoustic signal. However, the acoustic signal could be due to an internal volume transfer, such as a water shift.

This work was supported by the Grass Foundation.

Nicotinamide and other inhibitors of Poly-ADP ribose formation interfere with differentiation of Arbacia. CATHERINE M. BEARCE, PAUL M. YANG, KRYSTYNA FRENKEL, AND WALTER TROLL (NYU Medical School).

Poly-ADP-ribose (PADPR) was discovered seventeen years ago. It seems to be formed when chemical or physical DNA damaging agents break DNA strands. Ionizing radiation causes the induction of PADPR synthetase through the action of oxygen radicals and DNA breakage. PADPR has a putative role in initiating DNA repair through ligase action. We now show that PADPR appears to function in normal cell division since specific inhibitors of PADPR synthetase slow cell cleavage, thereby blocking differentiation in *Arbacia punctulata.* Two types of inhibitors have been described: (1) those which compete with NAD, *e.g.*, nicotinamide which is an inhibitor, while the closely related nicotinic acid is not. These two compounds are known as Vitamin B_3 and are nontoxic. (2) Those which limit the availability of adenosine, *e.g.*, thymidine. In the present study, we noted that active inhibitors of PADPR synthetase, (nicotinamide, benzamide, 3-aminobenzamide, thymidine), interfere with *Arbacia* development while their inactive congeners (nicotinic acid, benzoic acid, thymine) are without effect. We also tested a known oxidation product of thymidine formed by ionizing radiation: 5-hydroxymethyl-2'-deoxyuridine, and found it to be more effective than thymidine in interfering with *Arbacia* development. It may therefore inhibit PADPR synthetase more effectively than thymidine. Its base, 5-hydroxymethyl uracil was inactive even at much higher concentrations. The finding that nontoxic specific inhibitors of PADPR interfere with normal cell division supports the notion that PADPR may be a necessary component of cell division, perhaps through contributions to DNA repair. Thus, the inhibition of differentiation may be due to an accumulation of errors that have not been repaired by a normal poly ADP ribose initiated DNA repair.

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Thyroid control of seawater adaptation in Fundulus heteroclitus. CAROLYN J. BURDICK (Dept. of Biology, Brooklyn College, Brooklyn, New York), BARBARA A. TAYLOR, AND WILLIAM SPIELFOGEL.

The hormone(s) essential for seawater (SW) adaptation in euryhaline fish are open to question. We have investigated the role of thyroxine (T_4) as a SW osmoregulatory hormone in the euryhaline killifish, *Fundulus heteroclitus.* Plasma T_4 levels were measured by a sensitive radioimmunoassay. They were significantly higher in SW as compared to freshwater (FW) adapted fish. Plasma T_4, plasma chloride, and gill Na-K ATPase activity were monitored at intervals following transfer of fully adapted FW fish to SW. Plasma chloride levels increased steadily for 72 hours at which time plasma T_4 increased sharply to levels characteristic of fully adapted SW fish. The increase in T_4 was followed by a marked increase in gill Na-K ATPase activity and a return of plasma chloride to physiological levels. Transfer of FW fish to SW containing the antithyroid drug, thiourea, prevented plasma T_4 from increasing at 72 hours. Under these conditions gill Na-K ATPase activity remained at FW levels and plasma chloride remained elevated. These results suggest that T_4 has a major osmoregulatory role in the adaptation of *Fundulus heteroclitus* to SW, acting at least in part by activating branchial Na-K ATPase which is responsible for the maintenance of plasma electrolytes at physiological levels in long-term adapted SW fish.

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Ionic channels in cultured adenohypophyseal cells. GABRIEL COTA AND CLAY M. ARMSTRONG (Marine Biological Laboratory).

The whole cell variant of the patch clamp technique was used to characterize the voltage-dependent ionic channels present in primary dispersed, identified pituitary cells maintained in culture. This characterization is an initial step to study the effect of regulatory factors of secretion. Cells were dispersed by collagenase digestion from either: (1) the pars intermedia of adult male rats, composed of a single category of endocrine cells, melanotrophs, or (2) the anterior two-thirds of the rostral pars distalis of the killifish *Fundulus heteroclitus*, composed by prolactin cells. Cells were kept 1–8 days in culture following standard procedures. Experiments were carried out at 18–21°C on round, isolated cells 10–15 μm diameter. In most cases the holding potential was −80 mV. The standard external/internal recording solutions contained (m*M*): 135 NaCl, 5 KCl, 10 $CaCl_2$/120 K-glu, 20 KF, 20 NaCl, 2 $MgCl_2$ and 10 EGTA (pH 7.30). Eight ms-depolarizing pulses to +20 mV activated three major components of ionic currents in every pars intermedia cell investigated: a fast inward Na current followed by a delayed K current and, after repolarization, an inward Ca tail current which decayed in two phases, fast and slow. K currents tended to decline slowly during 250 ms pulses to membrane potentials more positive than +20 mV. Suppression of outward currents by replacing internal K with Cs and NMG unmasked two components of Ca current that could be distinguished by their activation, deactivation, and inactivation time courses, their voltage range of activation, their sensitivity to external Cd, and their rate of wash out. Rundown of Ca channels was slowed by addition of 3 mM Mg-ATP to the internal solution. A Ca-entry activated component in the outward current was detected by recording K currents in the absence of internal EGTA. A similar pattern of Na, K, and Ca currents was observed in rostral pars distalis cells. The following main differences in fish cells relative to rat cells were observed: a smaller contribution of the slow component to the Ca tail current, a faster wash out of fast deactivating Ca channels, and a faster decay of K currents during long depolarizations.

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K^+-selective microelectrode study of internally dialyzed squid axon: evidence for K^+-binding in axoplasm. C. N. FONG (Department of Physiology, University of Toronto) AND D. C. CHANG.

Axoplasmic potassium activity, $(a_K)_i$, was measured by K^+-selective microelectrodes in internally dialyzed squid giant axons. In a series of experiments we recorded $(a_K)_i$ and membrane potential, E_m, from axons bathed in artificial seawater (ASW) and internally dialyzed with a solution containing 400 m*M* KF. When the ASW and the internal dialysis solution were replaced by K^+-free ASW and K^+-free internal dialysis solution, respectively, there was a rapid fall of $(a_K)_i$ to an asymptotic value of approximately 3×10^{-3} and a depolarization of E_m.

These observations show that free axoplasmic K^+ can be rapidly depleted when the axoplasm is exposed to conditions where it is essentially in contact with an infinite volume containing no K^+ (*i.e.*, dialysis with K^+-free solution).

If the dialysis is stopped after $(a_K)_i$ has reached an asymptotic value, $(a_K)_i$ increases almost immediately. Since the axon continues to be bathed in K^+-free ASW this increase of $(a_K)_i$ is most likely due to K^+ release from some K^+ reservoir in the axon. From the increase of $(a_K)_i$ we estimate that this reservoir can release K^+ at a rate of approximately 10^{-10} moles^2/s l axoplasm.

Studies of the enzymic quenching of free radicals by cells. PETER GASCOYNE, JANE MCLAUGHLIN, RONALD PETHIG, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Electron spin resonance studies from this laboratory (Gascoyne *et al.* 1984, *Int. J. Quantum Chem: QBS* **11**: 309–314, and Pethig *et al.* 1985, *Proc. Natl. Acad. Sci. USA* **82**: 1439–1444) demonstrated that normal and transformed cells can quench a mixture of ascorbyl and semiquinone free radicals. The radical quenching was concluded to be associated with NAD(P)H enzyme activity characterized by an active sulfhydryl group, and the rate of quenching mirrored the extent of cell transformation. These studies have now been extended to characterize the nature and kinetics of the enzymes involved.

The radical scavenging activity was isolated using differential centrifugation of homogenized cells followed by gel chromatography, and a correlation between the radical quenching and glutathione reductase activity was observed. Glutathione reductase, in the presence of NADPH, can reduce the semiquinone radicals to their hydroquinone form, and a roughly ten-fold greater quenching activity has been observed for the closely related flavoprotein lipoamide dehydrogenase in the presence of NADH. These enzymes are also able to quench such stable free radicals as TEMPOL and PROXYL (Sigma). The flavin-adenine dinucleotide (FAD) moiety acts as an essential one-electron transfer mediator in the radical quenching. Our present studies are directed towards the investigation of other (FAD) oxido-reductase enzymes capable of exhibiting similar radical quenching activity, and their relevance to our previously observed differences of the radical quenching behavior of normal and transformed cells.

This work is supported by the National Foundation for Cancer Research, and we acknowledge the valuable collaboration with Drs. Chiu-Nan Lai and Frederick F. Becker of the M. D. Anderson Hospital and Tumor Institute, Houston.

Funduscopic appearance of the light and dark adapted eye of the living toadfish (Opsanus tau). DONALD A. GROVER AND SEYMOUR ZIGMAN (University of Rochester, Rochester, New York).

A technique to examine and photograph the ocular fundi of living marine species has been developed. Serial funduscopic photography allows documentation of time-related changes in the living eye. Serial morphological changes were noted in the living toadfish eye between the dark and light-adapted states. Anterior migration of epithelial pigment in the toadfish (*Opsanus tau*) retina during light and dark adaptation has already been shown histologically. Six toadfish (*Opsanus tau*) were dark-adapted in running seawater tanks in the dark for 12 to 21 h. Using ambient room light (two 150W, 130V Sylvania soft white incadescent lamps), the fish were light-adapted and the fundi periodically examined with a Frigi-xonix indirect ophthalmoscope with photographic capabilities. The fully dark-adapted eye revealed virtually no choroid or pigment epithelium detail, but instead there are greyish brown to silvery or chalky white, linear streaks radiating from the optic nerve into the far periphery. These appear to be at the level of the nerve fiber layer. Recognition of retinal blood vessels is difficult at this stage. The optic nerve is chalk white, and its border is indistinct from the streaks that blend with it. With light adaptation, the streaks tend to fade, and the underlying choroid and pigment epithelium develop a uniform brownish-orange hue. In some cases, the streaks are first replaced by a linear, silvery shagreen radiating from the optic nerve. In full light adaptation, the typical orange and black mottled appearance of the underlying pigment epithelium and choroid appears, and the optic nerve develops a pinker hue. In the fully-adapted fundus, a vague radiation of lines is superimposed on the cross-hatched mosaic of black dots which are not visible in the fully dark-adapted eye. Thus, with light adaptation, anterior melanin migration in the pigment epithelium absorbs stray light between the photoreceptors to sharpen vision.

Support: NEI, RPB, and University of Rochester (Mullie fund).

Exploring electrical currents through squid axoplasm. LIONEL F. JAFFE (Marine Biological Laboratory) AND ROBERT D. ALLEN.

We measured currents through the cut ends of three different preparations of extruded squid axoplasm or of whole axon sections immersed in buffer x—a medium simulating the cytoplasmic milieu. In all cases we used an external probe of 30 μm diameter vibrating at about 1 KHz. (1) Sections of extruded axoplasm were sucked into a capillary so as to protrude slightly from one end and be far inside the other. A steady current of 1.2 to 1.5 $\mu A/cm^2$ entered the protruding end regardless of whether this faced the former cell body or synapse. So these currents are somehow associated with the difference in milieu near the protruding and buried ends of the axoplasm rather than its inherent polarity. (2) Whole axon sections were fully immersed in buffer. Again, steady currents (of 0.1 to 0.8 $\mu A/cm^2$) entered the cut faces regardless of their polarity. However, these whole axon currents were presumably driven by their plasma membranes. Moreover, this first experiment suggests that the currents entering a cut facing the former cell body are greater—perhaps four-fold so—than those entering a synapse-facing cut. So the polarity of these whole axon sections may somehow affect the size of their face currents. (3) Finally, sections of extruded axoplasm were fully immersed in buffer. Again, currents (of up to 1.4 $\mu A/cm^2$) entered a soma-facing end; but—entirely unlike preparations 1 and 2—these fell off extremely rapidly with distance from the axoplasmic surface (halving every 6 μm instead of every 200 μm) and included a large quadrature component—characteristics of currents induced by the probe itself rather than ones inherent in the preparation.

Supported by the National Vibrating Probe Facility.

A newly reported disease of elasmobranchs: Aeromonas salmonicida *infection of the little skate,* Raja erinacea. Louis Leibovitz and Catherine T. Tamse (Laboratory for Marine Animal Health, Marine Biological Laboratory).

Little is known of the bacterial diseases of elasmobranchs. The first report of *Aeromonas salmonicida* infection of an elasmobranch, the little skate, *Raja erinacea,* is reported. Recurrent annual spring and summer epizootics of *A. salmonicida* infection were diagnosed and characterized in captive skates. Epizootics of the disease occurred during peak aquaria temperatures and greatest skate population densities. Early signs of the disease were sudden high percentage mortality, inactivity, abnormal swimming attitudes, and hemorrhagic skin lesions in affected skates. As the disease progressed, the daily percent mortality rates decreased. Chronic skin lesions appeared, including necrotic and ulcerative dermatitis, often associated with skin abrasions and lacerations. Early necropsy findings revealed discrete pinpoint and larger hemorrhages on the surface of pale visceral organs, including the heart, liver, serosal surfaces, and gills. Uncoagulated free blood could be found in the pericardial sac, the lumen of the gastrointestinal tract, and the ovary. Late during the course of the disease, small round white spots were noted on the surfaces and cross-sections of dark bile-stained livers, and swollen darkened spleens.

Histologically, the disease was characterized as a generalized bacterial septicemia and angiitis associated with intravascular bacterial proliferation, thrombosis, infarction, tissue hemorrhages, and necrosis.

Aeromonas salmonicida was isolated as pure cultures from infected skate tissues on serum enriched Brain Heart Infusion agar with added salt (1.15 percent). The organism was identified by biochemical and serological studies.

The disease was treated successfully by oral intubation of 50 to 75 mg of oxytetracycline per kilogram of fish body weight daily for five days or by intraperitoneal injections of 30 mg of oxytetracycline per kilogram of body weight on two successive days.

This study was supported in part by a grant from the Division of Research Resources, National Institutes of Health (P40-RR1333-05).

Calcium-stimulated secretion in sea urchin eggs: exocytosis proceeds in vitro *in the absence of other ions.* Jessica Liu and Joshua Zimmerberg (NIH, Bethesda, Maryland).

One class of hypotheses on the mechanism of exocytosis invoke transmembrane fluxes of ions or small electrolytes. Calcium may cause exocytosis by increasing the permeability of the granular membrane or by stimulating active transport. We used the isolated planar cortex of the sea urchin egg *Lytechinus pictus* (Vacquier 1975, *Dev. Biol.* **43:** 62–74) to study the ionic requirements for the exocytosis initiated by micromolar calcium. We also treated the membranes in the absence of calcium with a variety of agents known to make membranes permeable to small substances to see if they could mimic the effect of calcium.

Submicromolar calcium concentrations seem to be the only ionic requirement for exocytosis. Isolated planar cortices displayed a exocytotic reaction half-maximal at 0.1 μM free Ca^{++} in isotonic raffinose and sucrose solutions buffered with EGTA and $CaCl_2$. Contamination calcium in the sugar solutions was high enough to stimulate exocytosis in unbuffered solutions. Here, ionic contamination was estimated (as equivalent conductivity) at 100 μM NaCl.

Digitonin (14 μM) in artificial ooplasm did not cause either granule swelling or exocytosis. Entry of lucifer yellow, but not calcein, into granules was seen only after such treatment. Exocytosis ensued upon addition of 10 μM Ca^{++} to cortices first treated with digitonin. Higher concentrations of digitonin (50 μM) led to explosive granule lysis. Stabilization of granule cores with dextran increased the threshold for digitonin action. Ionophore treatment (100 μM valinomycin and CCCP, 662 units/ml nystatin, and 600 μM Amphotericin B) had no effect on either granule stability or calcium-triggered exocytosis.

Thus, calcium stimulates exocytosis here by mechanisms other than a permeability change in the granule membrane to substances with molecular dimensions less than $1.2 \times 1.4 \times 5.7$ nm. Higher digitonin concentrations which induce explosive lysis may act much as calcium does to swell the granule prior to fusion by making the granule permeable to higher molecular weight substances.

A non-trypsinlike enzymatic activity in the coelomic fluid of the sea urchin, Strongylocentrotus droebachiensis. James A. Marcum (Massachusetts Institute of Technology) and Jack Levin.

Enzymatic activity was detected, employing chromogenic substrates, in the cell-free coelomic fluid of the sea urchin, *Strongylocentrotus droebachiensis.* Enzymatic activity was not detected in preparations of washed coelomocytes derived from the coelomic fluid suggesting that the enzyme(s) is a constituent of the perivisceral fluid. Maximal amidolytic activity developed within 10 h from the time of coelomic fluid collection.

Studies involving dilution of fresh coelomic fluid or admixture of fresh fluid with activated protease(s), suggest that the enzyme(s) is a zymogen(s). Optimal activity was obtained between pH 8 and 9. Diisopropylfluorophosphate inactivated the amidolytic activity, indicating that the protease(s) contains an active center serine. Additional studies with a crude preparation of trypsin derived from gastric tissue of *S. droebachiensis* as well as a battery of protease inhibitors of trypsinlike enzymes, strongly suggest that the coelomic fluid enzyme(s) is not a gut protease. Importantly, in the absence of heparin, purified human antithrombin was a poor inactivator of the coelomic fluid protease(s), but in the presence of the glycosaminoglycan dramatically decreased the enzymatic activity. In contrast, inhibition of the gut enzyme *via* antithrombin was accelerated only marginally by heparin. Extracts from the epithelium, coelomocytes, muscle, perivisceral membrane, and gonads of the urchin did not activate the coelomic fluid proenzyme(s), while enzymatic activity was generated completely utilizing small concentrations of urchin gastric trypsin. Preliminary studies involving trauma during removal of the coelomic fluid suggest that injury to the organism may initiate activation of the enzymatic activity. Our results include the first demonstration of heparin-facilitated inhibition of a non-vertebrate protease *via* antithrombin and suggest that mucopolysaccharide-potentiated inactivation of enzymes, such as the mammalian intrinsic clotting factors, *via* a protease inhibitor appeared during evolution as early as the deuterostomes. The physiologic role of the protease remains unclear, but it may function in host defense mechanisms involving response to tissue damage.

This work was supported by a Frederik B. Bang Fellowship (Marine Biological Laboratory) awarded to J.A.M.

Electrophoresis studies of normal and transformed cells. JONATHAN PRICE, PETER GASCOYNE, JANE MCLAUGHLIN, RONALD PETHIG, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Measurements of the rate at which ascorbyl and semiquinone free radicals are quenched by Ehrlich ascites tumor cells can be used to determine the magnitude of cell-surface charge (Pethig *et al.* 1984, *Proc. Natl. Acad. Sci. USA* **81**: 2088–2091). To verify this, values of cell charge are being derived by measuring the electrophoretic mobility, and good agreement is obtained.

The electrophoresis studies have been extended to investigate the metabolic dependence of cell surface charge, differences between the mobility of normal and transformed cells, and the effects of exposing cells to externally generated free radicals. When suspended in phosphate buffered saline, Ehrlich ascites cells exhibit a steady mobility of $-1.09\ \mu ms^{-1}V^{-1}cm$ for at least 2 hours, after which there is a steady reduction over a period of several hours to a base-line level of $-0.8\ \mu ms^{-1}V^{-1}cm$. On exposing ascites cells to 40 μM and less 2,6-dimethoxy-quinone no change occurs in cell mobility, whereas exposure to 0.5 μM of the corresponding semiquinone radical causes the mobility to fall to the base-line level within minutes.

A temperature sensitive clone (6M2) of virally infected rat kidney cells exhibits a transformed phenotype at 33°C and a normal phenotype at 39°C. The electrophoretic mobility of the transformed 6M2 cells is observed to be $-1.4 \pm 0.08\ \mu ms^{-1}V^{-1}cm$ compared with the value of -0.95 for the normal phenotype. This 47% difference can be compared with the 4% decrease in mobility for human erythrocytes as the temperature is increased from 33°C to 39°C.

This work is supported by the National Foundation for Cancer Research, and we thank Dr. Chiu-Nan Lai of the M. D. Anderson Tumor Institute for supplying the 6M2 cells.

Characterization and comparative study of an atypical strain of Aeromonas salmonicida *isolated from the little skate,* Raja erinacea. CATHERINE T. TAMSE AND LOUIS LEIBOVITZ (Laboratory for Marine Animal Health, Marine Biological Laboratory).

Aeromonas salmonicida, once considered a universal freshwater and anadromous salmonid pathogen, has been found recently in new marine and freshwater hosts (sablefish, Atlantic cod, eel, goldfish, carp). This study characterized an atypical *A. salmonicida* strain isolated from laboratory-maintained little skates (*Raja erinacea*) during an epizootic at the Marine Biological Laboratory, Woods Hole, Massachusetts. This is the first reported isolation of *A. salmonicida* from an elasmobranch, *R. erinacea.* The Woods Hole, typical salmonid and other atypical strains were compared.

Morphologically, twenty-four hour colonies of the skate isolates grown on Brain Heart Infusion (BHI) agar with 1.15% NaCl supplement were small, round, 0.5–1.5 mm-diameter, and were convex with entire margins. The typical brown, water-soluble pigment of *A. salmonicida* was also produced after 24–48 hours incubation at room temperature (20°C). All the isolates were gram-negative, non-motile, medium-sized rods with rounded ends. Optimum growth in BHI broth was observed at 0% and 3% NaCl. No growth occurred between 5% and 10%.

Biochemically, the Woods Hole and typical strains were similar in failing to produce indole and acetoin, reducing nitrate, and fermenting glucose, galactose, maltose, mannitol, and trehalose but not sucrose, inositol, and xylose. The skate isolates differed notably in having alkaline arginine decarboxylase reactions, failure to liquefy gelatin, and ability to ferment arabinose but not salicin. All antibiotic sensitivity tests yielded consistent results with those of the typical *A. salmonicida* isolate.

Serologically, microslide and tube agglutination tests revealed a positive antigenic relationship between the Woods Hole isolate and the typical and atypical *A. salmonicida* isolates from non-elasmobranch sources.

Biochemical data presented indicate that the marine skate isolates belong to the atypical group of *A. salmonicida* but are serologically related to both typical and atypical isolates. Individual biochemical differences also exist between atypical marine and freshwater *A. salmonicida* isolates.

This study is supported in part by a grant from the Division of Research Resources, National Institutes of Health (P40-RR1333-05).

High colloid osmotic pressures inhibit dispersal, the second stage of exocytosis in sea urchin eggs. MICHAEL WHITAKER AND JOSHUA ZIMMERBERG (NIH, Bethesda, Maryland).

Exocytosis is a two stage process. The first stage comprises granule swelling which leads to the fusion of the granule membrane with the plasmalemma. The second stage involves an explosive dispersal of the granule contents (Zimmerberg and Whitaker 1985, *Nature* **315:** 581–584). We have shown that preventing water movement during the first stage of exocytosis using solutions of low molecular weight substances prevents fusion. We now show that solutions of higher molecular weight substances (colloids) prevent the second stage.

We find that solutions of dextran [10,000 and 500,000 weight-average molecular weight (M_{av})], and polyethyleneglycol (3350 M_{av} and 20,000 M_{av}, but not 1450 M_{av}) of 0.25 osmol/kg colloid osmotic pressure (COP) in seawater of 0.93 osmol/kg prevent exocytosis as judged by observation of cortical granules in *Lytechinus pictus* eggs activated by the calcium ionophore A23187. These solutions do not inhibit fusion, since the membrane capacitance increase brought about by fusion of the granule membrane with the plasmalemma occurs. This indicates that the cortical granule contents behave much like a gel filtration bead, excluding molecules with molecular weights greater or equal to 3350.

The COP required to prevent granule dispersal depends upon the concentration of divalent cation in the external solution. In the absence of divalent cations a COP of 0.9 osmol/kg was required. Inhibition of dispersal by 0.25 osmol/kg COP was unaffected by monovalent cations at concentrations of up to 450 mM, indicating that the effect of divalent cations is due to binding rather than screening of negative charge. Replacing chloride by sulfate had no effect.

Thus, the granule contents behave as a divalent cation-stabilized gel during exocytosis. These results lead us to believe that alterations in the affinity of the granule interior for water may be responsible for the initial swelling of the granule which leads to membrane fusion.

Properties and functions of near-UV absorbing pigments in marine animal lenses. SEYMOUR ZIGMAN, TERESA PAXHIA, AND WILLIAM WALDRON (University of Rochester, Rochester, NY).

The sunlit environment provides intense near-UV radiation (295–400 nm) to the earth's surface which penetrates through seawater with only partial attenuation. The visual apparatus of certain animals is adapted to such environmental radiant energy by including near-UV filters in the ocular lens. Deep-swimming fish lack such lens pigment so as to utilize all available light for optimal vision. The lenses of surface fish filter out short wavelength radiant energy to minimize chromatic aberration (misfocusing of short wavelengths) and to maximize contrast (against the blue sky). Thus, the lenses of many shallow swimmers contain short wavelength chemical filters (or pigments) similar to those of many diurnal land animals. There are two major near-UV absorbing pigments in the lenses of assorted teleosts and squid. These pigments absorb maximally at 320 nm (I) or at 360 nm (II), and are low molecular weight, dialyzable, water-soluble compounds. I is more polar than II, as determined by C_{18}-porasil cartridge chromatography using H_2O and methanol as solvents with stepwise gradient elution. Lens pigments are present as follows: mainly I in scup (*Stenotomus versicolor*), butterfish (*Poronatus triacanthus*), and squid (*Loligo peleii*); mainly II in cod (*Microgadus tomcod*), sea robin (*Prionotus carolinus*), and sea bass (*Centropristes striatus*); I and II equivalent to goosefish (*Lophius americanus*), flounder (*Paralichthys dentatus*), and bluefish (*Ponatomus saltatrix*); and none in dogfish (*Mustelus canis*), skate (*Raja erenacea*), toadfish (*Opsanus tau*), and tautog (*Tantoga onitis*). While the chemical natures of I or II are not known, they resemble naturally occurring tryptophan oxidation products (*i.e.:* kynurenines). Aerobic near-UV photooxidizing conditions only altered II and not I. In general, deep swimming fish do not contain near-UV pigments in the lens, while shallow swimmers contain mostly II (320 max.), and surface swimmers contain mostly I (360 max.). These findings suggest that near-UV absorbing lens pigments enhance the visual process in the eyes of marine animals active in bright sunlight.

Support: NEI, RPB, and University of Rochester (Pledger fund).

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CHANGES IN BEHAVIOR AND OCELLAR STRUCTURE DURING THE LARVAL LIFE OF SOLITARY ASCIDIANS

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ABSTRACT

Larvae of *Ciona savignyi* Herdman change swimming behavior during the course of development. Newly hatched larvae swim upward by negative geotaxis, accumulating beneath the water surface. Thirty minutes after hatching, the two-dimensional spread becomes more regionally restricted and the aggregated pattern looks like a swarm of mosquitoes. One and a half hours after hatching, larvae become photoresponsive, swimming upward for a short time immediately after shading. Meanwhile, the duration of swimming induced by shading becomes longer. Two and a half hours after hatching, larvae are weakly photonegative. Three and a half hours after hatching, swarming is abolished and photonegativity is much stronger than the previous stage.

The larval ocellus differentiates after the tadpole hatches. In newly hatched larvae, the flat pigment cell contains sparsely scattered pigment granules. Several short tubular membranes derived from ciliary endings of photoreceptor cells are irregularly arranged. One hour after hatching, the pigment cell becomes roughly L-shaped. The originally tubular membranes become paddle-shaped and increase in number and size. The pigment cell then assumes a V- or J-shape and becomes loaded with densely packed pigment granules. Some of the paddle-shaped membranes are arranged into lamellae and increase greatly in number and length. Three and a half hours after hatching, the ocellus becomes fully differentiated.

Using morphometrical parameters as regards the size of photoreceptor endings and the disposition of pigment granules, we show that changes in photic behavior coincides roughly with the course of differentiation of the ocellar elements.

INTRODUCTION

Larvae of sessile marine invertebrates are generally pelagic and respond to ecological factors in species-specific ways, by which they reach the substratum. One of the predominant ecological factors involved in larval settling is light (Thorson, 1964, for review). Although the morphology of the photoreceptor systems of invertebrates has been studied widely (Eakin, 1970, for review), works on the dynamic relationship between changes in photic behavior and photoreceptor morphogenesis during the larval life are rare (Young and Chia, 1982, in a polychaete; Chia and Koss, 1983, in an opisthobranch). In ascidians, several investigators (Grave, 1920; Mast, 1921; Woodbridge, 1924; Crisp and Ghobashy, 1971) explored larval behavior, while others (Dilly, 1961, 1964; Eakin and Kuda, 1971; Barnes, 1971) studied the ultrastructure of fully differentiated larval ocelli. The work of Barnes (1974) on "embryos" of *Amaroucium constellatum* is the only one to deal with the differentiation of the ocellus. Here we report that behavioral changes occurring during the course of solitary ascidian

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larval life roughly correspond in time to photoreceptor differentiation. A substantial part of the present work has appeared in abstract form (Kajiwara and Yoshida, 1983).

MATERIALS AND METHODS

Specimens of *Ciona savignyi* Herdman (Hoshino and Nishikawa, 1985) were reared on flower pots which were hung from the pier of the Ushimado Marine Laboratory into the sea. Gravid individuals were maintained in running seawater at 18–23°C and continuously illuminated with two 20 w fluorescent lamps (50 cm high from animals) for 1–10 days to prevent uncontrolled spawning (Lambert and Brandt, 1967). Additional observations were made using *Ascidia sydneiensis sames* Oka. Unless specified, however, the following descriptions concern *C. savignyi.* In both cases, gametes were obtained surgically from gonoducts. Cross-fertilized eggs were maintained at room temperature (21°C) after several washes with a large volume of filtered seawater. Hatching occurred nearly synchronously about 14 and 16 h after fertilization in *C. savignyi* and *A. sydneiensis samea,* respectively. Larvae which were collected within 15 min after the first larva hatched are referred to as "newly hatched larvae."

Larval behavior was studied as follows. A transparent plastic trough, 8 cm square and 5 cm deep, was placed under ambient room light enhanced with window light, the intensity of which (1300 lux) was adjusted by window-blinds. One side of the trough was parallel to the window such that a photic gradient was created across the trough, decreasing from the window side toward the inner wall. This arrangement was advantageous for examining the presence or absence of shadow reflexes as well as positive or negative phototaxis, because simply placing a black plate on the window side not only reduced the light intensity to 150 lux but also reversed the existing photic gradient across the trough. This reduction will be called "shading."

Distribution patterns of larvae were photographed at 15 min intervals from 15 min through 5 h after hatching. To record horizontal and vertical distribution patterns of larvae, two cameras were triggered simultaneously from above as well as from the side of the trough. The side camera was parallel to the window and a strobe flash was shone from the side opposite the window. The side camera looks beneath the water surface, recording not only the side view of the trough but also the reflected images of larvae just below the water surface. However, such an artifact was assumed to be immaterial for assessing changes in the distribution pattern of larvae in the middle and the deeper levels, and hence the presence or absence of their upward movements.

The trough contained 3 cm of seawater containing 4–6 larvae per ml. After gentle stirring, a transparent lid was put on the trough to avoid any air-borne disturbance to the water surface and the seawater was left undisturbed for 14 min until the first recording ("Initial" in Figs. 1, 3–5) was made. Shading commenced 5 s after the initial recording, and the distribution patterns were recorded at 2, 5, 10, 15, and 30 s after shading. The seawater was then stirred and the lid replaced until the Initial of the next recording period.

Differentiation of the structures associated with the cerebral vesicle was followed electron microscopically. Larvae were fixed at room temperature for 2 h in 2.5% glutaraldehyde buffered to pH 7.4 by 0.1 mol sodium cacodylate containing 0.4 mol sucrose. The fixed tissues were washed by three 15-min changes of the same buffer, post-fixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol, penetrated with propylene oxide, and embedded finally in an epoxy resin (TAAB embedding resin). Thin sections cut on a Porter-Blum MT-2 ultramicrotome were stained with alcoholic uranyl acetate and lead citrate, and examined with a Hitachi H-500H electron microscope.

Using an image analysis system (MOP-Videoplan, Kontron Electronic Group), various morphological parameters of photoreceptor endings and pigment cells were measured on enlarged images of EM negatives.

RESULTS

Larval behavior

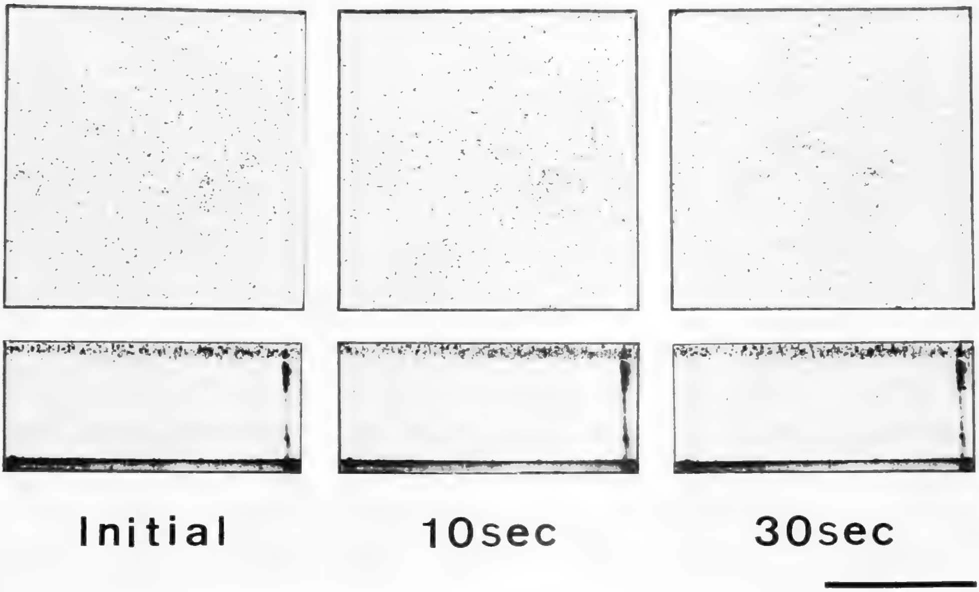
Using the behavioral pattern as a criterion, larval development was divided into five stages. Typical examples from 25 series of experiments are shown in Figures 1 through 5.

Stage I: until 15 min after hatching

Immediately after hatching, larvae move sporadically on the bottom of the trough by tail twiches. They soon begin to swim circularly at the substratum by brief bursts of tail beating. A few minutes later, the swimming increases in duration as well as in distance. In combination with negative geotaxis, larvae reach the water surface and swim circularly (Fig. 1). When observed from the front, these larvae appear to rotate clockwise with respect to their anterior-posterior axis. Stage I larvae of both *C. savignyi* and *A. sydneiensis samea* do not respond to changes in light conditions.

Stage II: Around 1 h after hatching

Larvae of both species are still indifferent to light. A notable phenomenon that begins to appear at this stage is the tendency to aggregate into a column which looks



FIGURES 1–5. Larval distribution patterns in a square trough photographed from above (upper row) and from a side (lower row), showing temporal sequence of changes of larvae at different stages. In Figures 1 and 3–5, shading was from the right side after initial recording and time after shading is given under each photograph. Scale: 4 cm.

FIGURE 1. The distribution pattern of Stage I larvae (15 min after hatching).

like a swarm of mosquitoes. This type of behavior starts 30 min after hatching in *C. savignyi* and 1 h in *A. sydneiensis samea.* The sequence of swarm formation is shown in Figure 2. Close observation of individual larvae revealed that while swimming beneath the water surface, they often stopped swimming and sank passively. The frequency of larval stopping was increased when two larvae collided with each other. After sinking to an indeterminate depth, larvae resumed upward swimming slightly outside the original sinking line. In this way, the larvae form first a ring pattern when viewed from above (2.5 min in Fig. 2) which becomes smaller and smaller until a single column is formed at the end (4.5 min in Fig. 2).

Stage III: 1.5 h after hatching

Larvae begin to show a shadow reflex, swimming upward within 2 s after shading. Note that in the side view of the 10 s recording in Figure 3, the number of larvae on the bottom is much reduced. Negative phototaxis is not apparent as yet, so that the distribution pattern in the trough remains the same as Stage II to the end of the recording period (30 s in Fig. 3).

Stage IV: 2.5 h after hatching

Larvae still form the swarm as before but now the size increases because the degree of aggregation has decreased (Initial in Fig. 4). A notable difference from the preceding stage is that the swarm disperses in about 10 s after shading. The massive shift towards the darker side (rightside in 10 s in Fig. 4) during shading indicates the onset of photonegativity. 30 s after shading, a few larvae begin to sink slowly. They form a swarm again after about 10 min when left undisturbed. Larvae of *A. sydneiensis samea* also become negatively phototactic about 3 h after hatching.

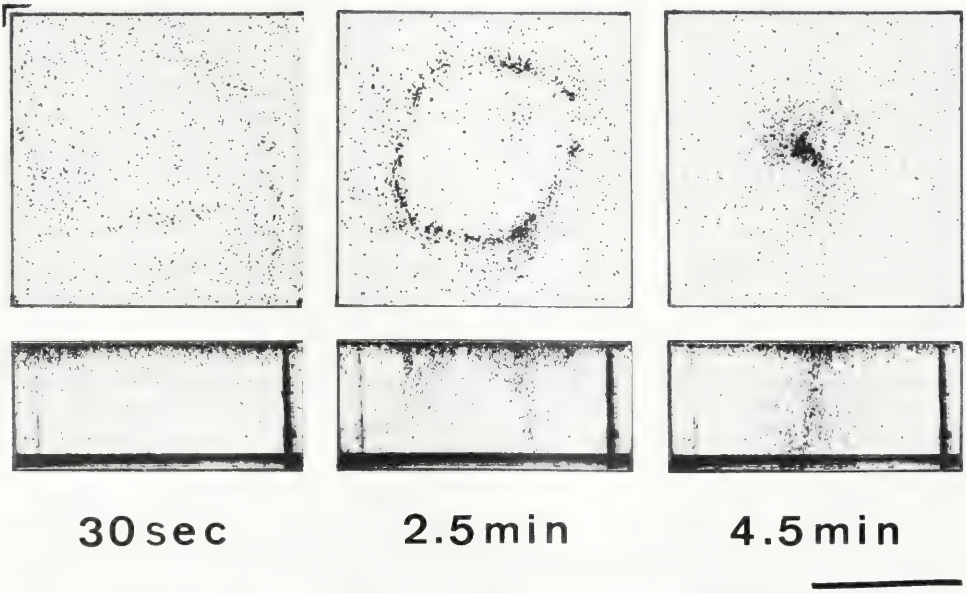


FIGURE 2. The sequence of swarm formation which occurs between Stage II and IV. This experiment was done with Stage III larvae. The time after stirring seawater is given under each photograph.

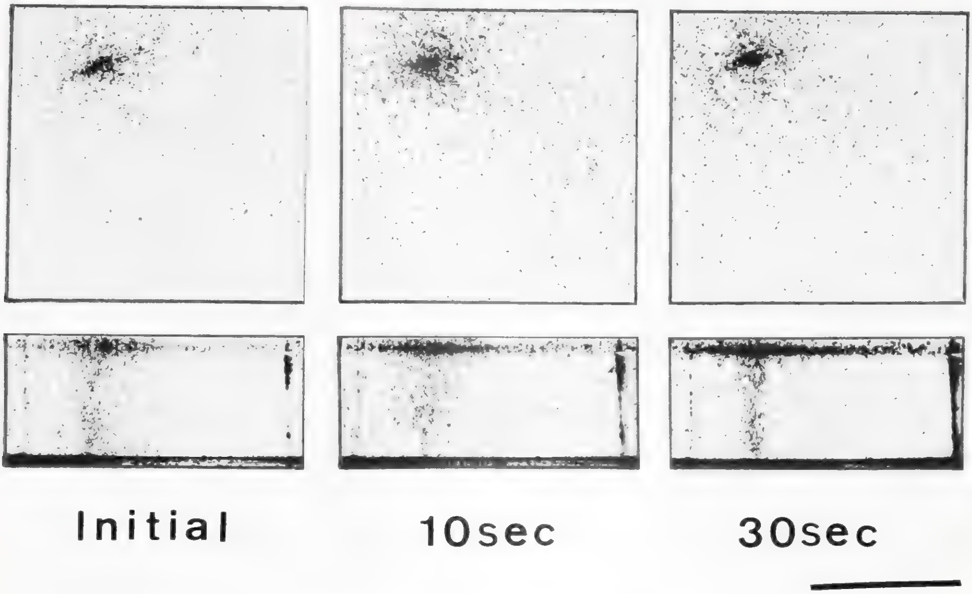


FIGURE 3. The distribution pattern of Stage III larvae (1.5 h after hatching).

Stage V: 3.5 h after hatching

As shown in the initial record in Figure 5, larvae no longer form the swarm but the majority are scattered on the left half, the side opposite the window due to the

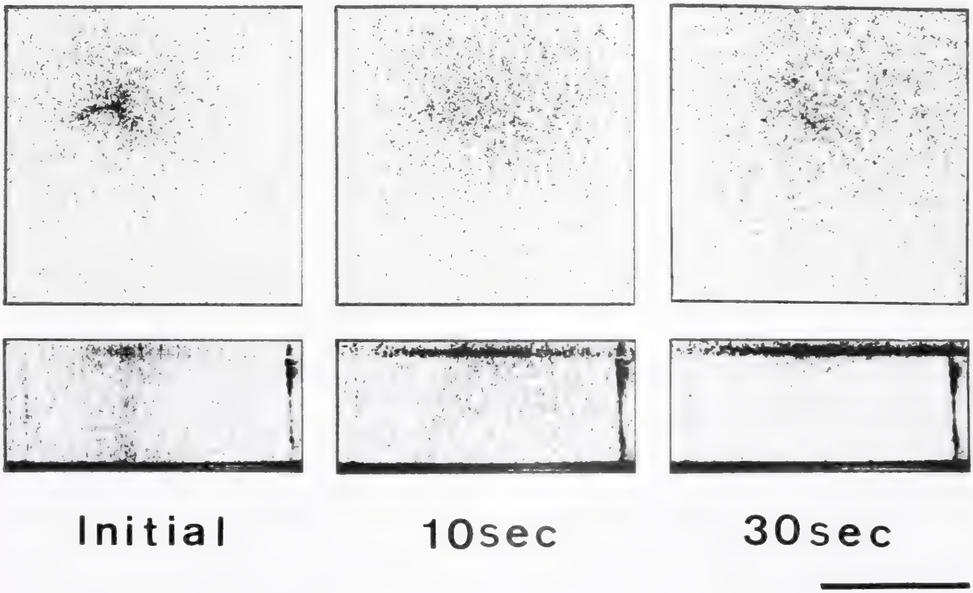


FIGURE 4. The distribution pattern of Stage IV larvae (2.5 h after hatching).

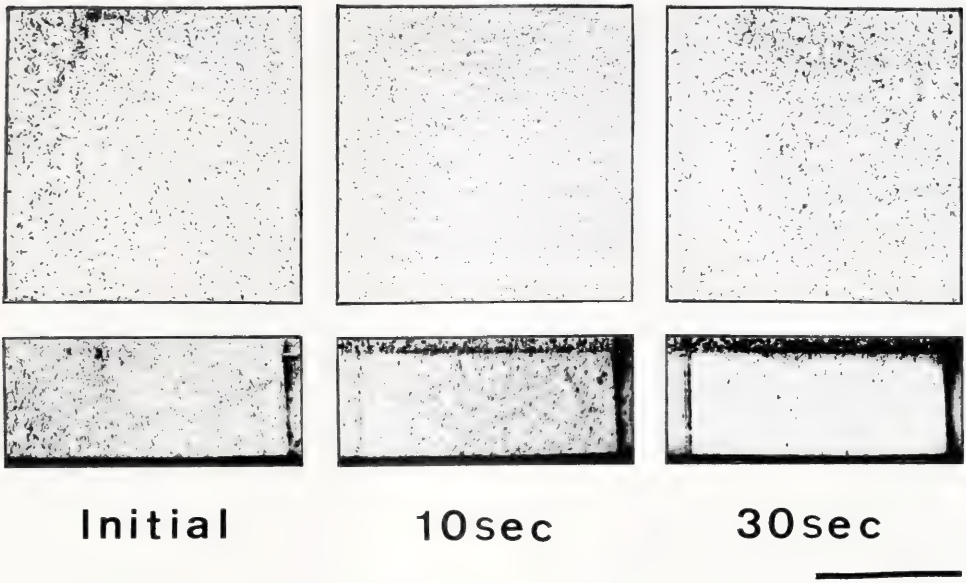


FIGURE 5. The distribution pattern of Stage V larvae (3.5 h after hatching).

photonegativity. Immediately after shading, larvae begin swimming upward as well as to the darker side (right side in 10 s in Fig. 5). Thus, the photonegative response is stronger than before. By 15 s after shading, the majority reach the water surface and a few begin to sink again 30 s after shading.

Differentiation of ocellus

The structure of the fully differentiated ocellus has been described by Dilly (1961, 1964), Eakin and Kuda (1971), and Barnes (1971), so that a brief description is sufficient here. In both species examined, the ocellus is located on the right-posterior wall of the cerebral vesicle, pointing ventro-laterally (Fig. 6a, b). Each ocellus consists of three components (Fig. 6c). (1) One cup-shaped pigment cell which is loaded with membrane-bounded melanin granules (Whittaker, 1973, 1979) appears as a whole mass, V-, or J-shaped in cross sections. (2) About 15–20 photoreceptor cells line up on the lateral side of the pigment cell. Each cell extends a narrow process through the pigment cell giving rise to about 25 lamellae from the ciliary projection within the cup lumen. (3) Three lens cells are arranged in a row and each of them contains a large lens vesicle bordered by mitochondria.

To investigate whether there are any changes in the morphological differentiation of the ocellus corresponding to changes in larval behavior, materials were fixed at the 5 stages defined by behavioral criteria. Morphometric analysis included the following five parameters, using transverse profiles of five ocelli for each stage. For photoreceptive elements in each ocellus, the cell whose lamellae were greatest in number and cut longitudinally was chosen. In this cell the number of lamellae was counted and the length of these lamellae was measured. For pigment cells, the cell profile showing the largest cup lumen was chosen and the following determined: (1) size of pigment granules, (2) ratio of the total area occupied by pigment granules to that of the pigment cell (nuclear zone excluded), and (3) ratio of the total area occupied by pigment granules

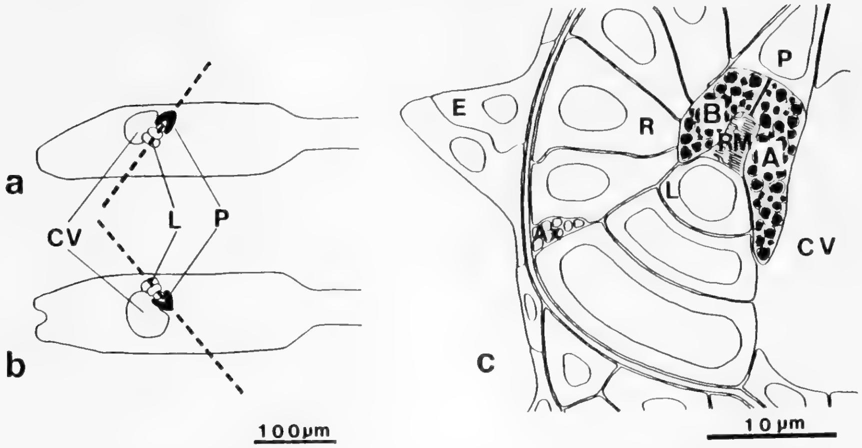


FIGURE 6. Semi-diagrammatic representation (roughly to scale) of the gross appearance of the ocellus, constructed from serial LM and EM sections. a and b: Lateral and horizontal profiles, respectively. c: An expected profile when the plane of the section passes the oblique broken lines drawn in a and b. Ax: axons of photoreceptor cells, CV: cerebral vesicle, E: epidermal cell, L: lens cell, P: pigment cell, R: photoreceptor cell, RM: photoreceptive membranes.

in the lateral side of the cup (B in Fig. 6c) to that in the medial side (A in the same Figure).

Stage I: until 15 min after hatching

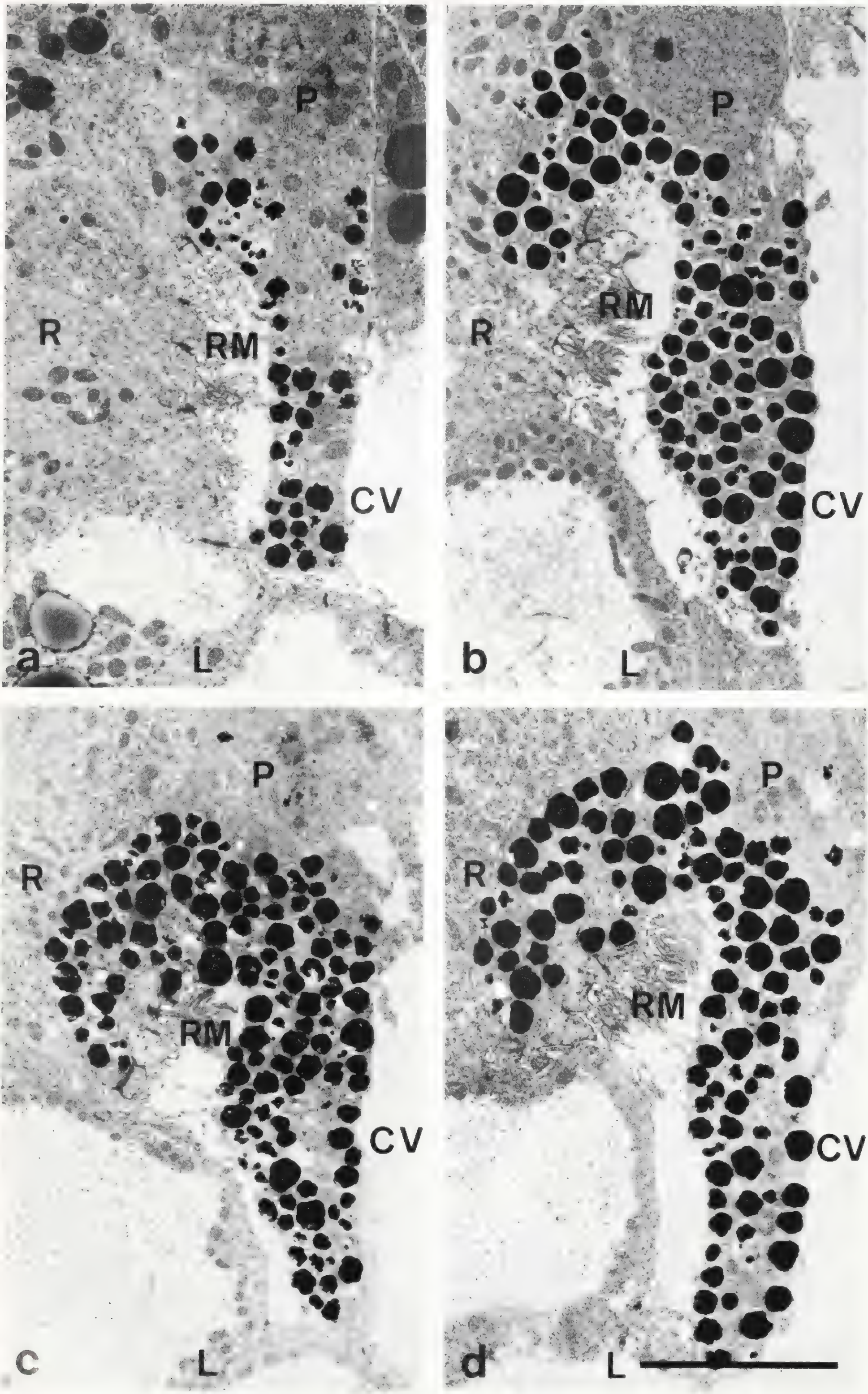
The pigment cell does not extend the B-portion in Figure 6c (Fig. 7a). The pigment granules are small in size (0.15 ± 0.04 µm; n = 382, Fig. 10b) and in number so that the area covered by the pigment granules occupies 31.3 ± 6.9% of the pigment cell cytoplasm. Some of the membranes derived from the apex of the photoreceptor cell are tubular as revealed by the cross-section in Figure 9a. The number of lamellae arising from single cells ranges from 9 to 16. They are short (0.47 ± 0.07 µm; n = 60, Fig. 10a) and irregularly oriented (Fig. 8a).

Stage II: 1 h after hatching

The pigmented area starts to extend toward the lateral side (B-portion in Fig. 6c) of the cerebral ganglion. Pigment granules become larger (0.19 ± 0.02 µm in diameter; n = 483, Fig. 10b) and occupy a larger portion of the pigment cell (42.3 ± 5.8%, Fig. 10b). The lamellae from the photoreceptor cell increase in number, ranging from 16 to 28, from single cells and in length (0.58 ± 0.06 µm; n = 92, Fig. 10a).

Stage III: 1.5 h after hatching

The growth of the B-portion of the pigment cell continues so that the pigmented area now appears roughly J-shaped (Fig. 7b). Pigment granules become larger (0.26 ± 0.04 µm in diameter; n = 379, Fig. 10b). Membranes arising from the photoreceptor projections are transformed into a paddle-shape (Fig. 9b). The number of lamellae from single cells ranged from 19 to 23 and the average length was 0.62 ± 0.07 µm (n = 109, Fig. 10a).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Stage IV: 2.5 h after hatching

The extension of the B-portion nears completion so that the pigmented area takes a V-shape form. At this stage, narrow processes of the photoreceptor cells are surrounded by the pigment cell. As shown in Figure 10b, the average diameter of the pigment granules (0.24 ± 0.02 μm; n = 672) and the ratio of the pigmented area to the cytoplasm (48.3 ± 2.6%) are not much different from the previous stage, but the ratio of the pigmented area in B to that in A is markedly increased, from 26.4 ± 4.4% in Stage III to 55.5 ± 11.0% in Stage IV (Fig. 10b). It is also noteworthy that although the number of lamellae arising from single cells is not much increased (range: from 20 to 28), their length (0.88 ± 0.07 μm; n = 120) are approaching the maximum value in the next stage (Fig. 10a).

Stage V: 3.5 hours after hatching

The larval eye in this stage (Fig. 7d, Fig. 8d) is fully differentiated. Morphometrical values of all five parameters fall within the standard deviation of those in the previous stage (Fig. 10a, b).

DISCUSSION

Sessil marine invertebrates are found in restricted zones of rocks or sand beaches. Pelagic larvae disperse themselves and finally choose the best-fitted substratum for future life. This adaptive strategy is achieved through dynamic changes in response to various ecological factors. The temporal sequence of changes in the larval behavior of the ascidian, *C. savignyi,* may be summarized as follows (refer to Fig. 10a, inset). (1) Newly hatched larvae swim up geonegatively and are indifferent to light. (2) Thirty min after hatching, larvae begin to form a swarm under all light conditions. (3) One and a half h after hatching, shadow reflexes that last until metamorphosis begins to appear. (4) Two and a half h after hatching, larvae become negatively phototactic though still weak. (5) Three and a half h after hatching, larvae no longer form the swarm and the shadow reflex and photonegative response are strongest. Metamorphosis occurs at any time between 5 h and 2 weeks after hatching.

From these behavioral patterns in the experimental trough, we can predict the larval behavior in the field: newly hatched larvae swim up to the water surface and are dispersed with water currents as they repeat up and down excursions in the upper stratum. Later, larvae become photonegative and occasionally stop swimming, sinking down to a deeper layer. Upon reduction in light intensity, they swim toward a darker area as well as upward. In this way, they will tend to settle on under-surfaces of the substrata in their late stages.

The behavioral patterns described above appear to be typical of solitary ascidians. Similar behaviors were also described in *Styela partita* (Grave, 1941, 1944). However, the larvae of many compound ascidians show positive phototaxis for a short period in the early stage, and soon it changes to negative phototaxis (*Amaroucium constellatum:* Grave, 1920, Mast, 1921; *Amaroucium pellucidum:* Mast, 1921; *Botryllus schlosseri:* Woobridge, 1924; *Diplosoma listerianum:* Crisp and Ghobashy, 1971).

It may be expected that the functional differentiation of ocelli would correspond

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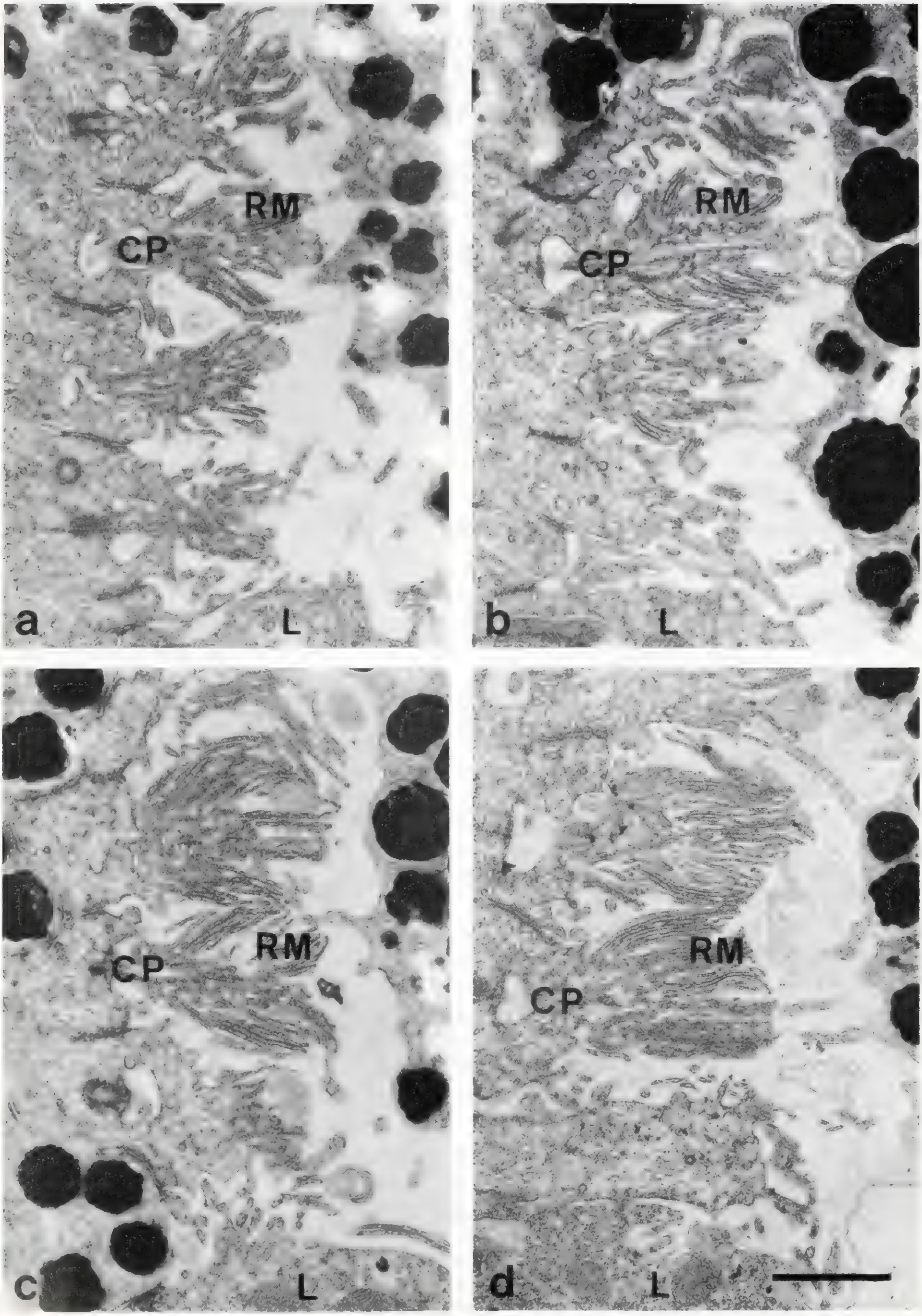
FIGURE 7. Changes in the shape of pigmented area during larval life. a: Stage I, b: Stage III, c: Stage IV, d: Stage V. CV: cerebral vesicle, L: lens cell, P: pigment cell, R: photoreceptor cell, RM: photoreceptive membranes. Scale 5 μm.


RM
CP
a
L
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RM
c
L
RM
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FIGURE 8. Changes in the ultrastructure of the photoreceptive membranes during larval life. a: Stage I, b: Stage III, c: Stage IV, d: Stage V. CP: ciliary projection, L: lense cell, RM: photoreceptive membranes. Scale: 1 μm.

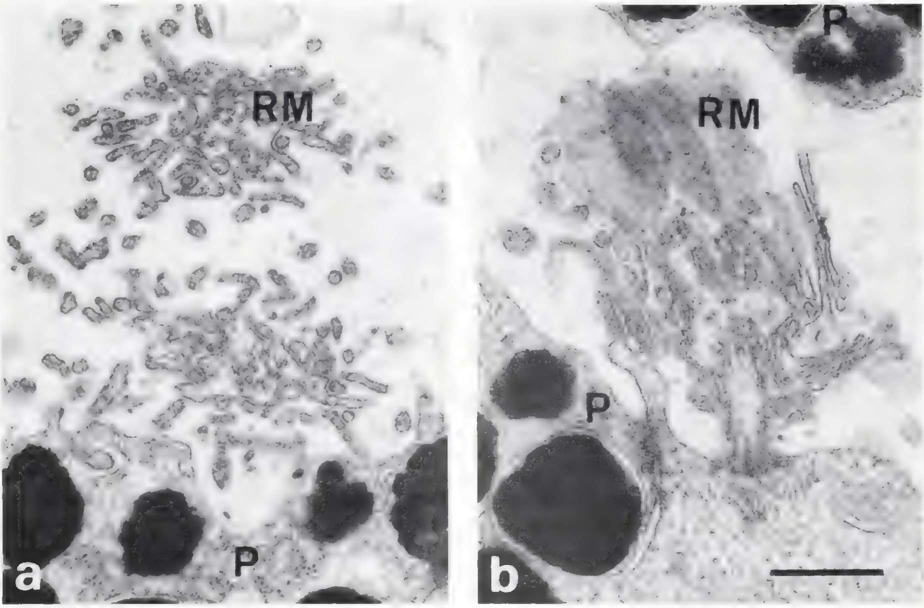

FIGURE 9. Magnified profiles of the distal part of photoreceptor cell. a: Stage I. b: Stage III. P: pigment cell, RM: photoreceptive membranes. Scale: 0.5 μm.

to the development of photic responses. Although the ultrastructure of ocelli has been studied widely in differentiated larvae (Dilly, 1961, 1964; Barnes, 1971; Eakin and Kuda, 1971), the work of Barnes (1974) on "embryos" of *Amaroucium constellatum,* a compound ascidian, was the only one that followed the differentiation of the ocellus. Our observations confirmed and extended those of Barnes by introducing morphometrical aspects. Differentiation of photoreceptive membranes and the pigment cell proceeds rapidly within 3 h. One and a half h after hatching (Stage III) the larvae become responsive to a shadow. Although we could not ascertain the number of newly recruited photoreceptor cells between Stage I and Stage III, the increase in surface area of the photoreceptive membrane as estimated by the number of lamellae from single cells (from 12.0 to 21.8) and their length (from 0.47 to 0.62 μm) should account for the functional maturation of the presumptive photoreceptor cells.

The negative phototaxis then appearing from Stage IV onward is accompanied by a more than two fold increase in the ratio of the pigmented area in the B-portion to that in A-portion, resulting in both of the lateral sides of the ocellar lumen becoming loaded with pigment granules. This pigment disposition may aid the photoreceptors in sensing the direction of the light source. The arrangement of lamellae perpendicular to the incident light coming through the lens system may also be advantageous for photosensitivity. Considering that ocelli of newly hatched larvae which are indifferent to light are already equipped with the three major components (a single pigment cell, photoreceptor cells and three lens cells) of the ocellus, maturation of the central nervous network may also be needed for performance of complex behavioral responses.

Although swarm formation may only be an experimental phenomenon, its appearance and disappearance could be taken as a manifestation of dynamic changes in the developing nervous mechanisms at the middle of larval life. For the swarms to be formed, mechanoreceptors such as the pressure receptors in the cerebral vesicle

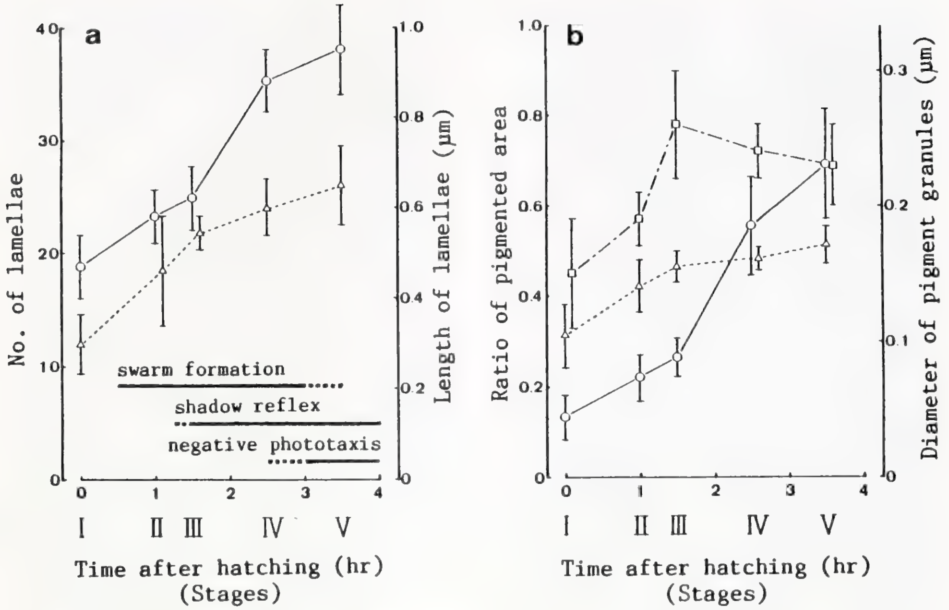

FIGURE 10. Changes in morphometric parameters of photoreceptive membranes (a) and pigment granules (b) during larval life (abscissae: time after hatching in h and corresponding stages). a: Triangles show the number of lamellae arising from single cells (ordinate on the left) and circles, the length of lamellae (ordinate on the right). Inset shows the temporal sequence of the appearance and disappearance of three behavioral criteria (dots: weak responses). b: Squares show the diameter of pigment granules in cross-sections (ordinate on the right), triangles, the ratio of the pigmented area to the cytoplasma and circles, the ratio of the pigmented area in the B-portion in Figure 6c to that in the A-portion (for the latter two, ordinate on the left).

(Eakin and Kuda, 1971; Reverberi, 1979) and sensory cilia in the tail epidermis (Torrence and Cloney, 1982) could be involved in sensing the depth and the contact with neighboring larvae, respectively. The fact that the swarm disappears as metamorphosis approaches may suggest that an integrating mechanism which has been concerned with mechanical stimuli during the middle of larval life may be taken over by photosensory mechanisms which will be important for seeking a substratum to settle.

The sensory and neural mechanisms involved in larval behaviors of solitary and compound ascidians remains to be studied in more detail.

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BEHAVIORAL CORRELATES OF CIRCADIAN RHYTHMS IN THE *LIMULUS* VISUAL SYSTEM

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ABSTRACT

A clock in the brain of *Limulus* generates circadian rhythms in retinal sensitivity. We examined the relation between behavioral responses to light and circadian changes within the visual system. Our first experiment recorded unconditioned movements of the telson (tail) elicited by a constant level of illumination of the lateral eyes at different times of the day while an animal remained in the dark. Under these conditions, tail movements followed the same pattern of response as the electroretinogram (ERG) recorded from the lateral eyes. That is, the probability of tail movement was directly proportional to the amplitude of the ERG and both exhibited a circadian rhythm. In the second experiment we conditioned the reflexive movements of the tail and gills by pairing illumination of the lateral eyes with an aversive stimulus, and then measured the level of illumination necessary to elicit responses at different times of the day. Results show that animals maintained in darkness are about 10 times more sensitive at night than during the day. The day-night change in visual sensitivity measured behaviorally is consistent with that measured physiologically (Barlow *et al.,* 1980). The daily rhythm of visual performance could thus be attributed to the known rhythm in retinal sensitivity generated by a circadian clock located in the brain.

INTRODUCTION

The visual system of the horseshoe crab, *Limulus polyphemus,* exhibits circadian rhythms in sensitivity. Illumination of the lateral eyes elicits larger receptor potentials, larger ERGs, and higher optic nerve discharges at night than during the day (Barlow *et al.,* 1977; Kaplan and Barlow, 1980). Such high nighttime responses result from changes in lateral eye structure (Barlow *et al.,* 1980), excitation (Barlow *et al.,* 1977), inhibition (Batra and Barlow, 1982), noise (Kaplan and Barlow, 1980), and metabolism (Chamberlain and Barlow, 1979, 1984) mediated by the efferent neural output of a circadian clock located in the brain (Barlow, 1983).

What behavior is served by such intricate cellular mechanisms for increasing retinal sensitivity at night? *Limulus* mates primarily at night (Cavanaugh, 1975; Howard *et al.,* 1984), and recent field observations indicate that vision plays a role in this behavior (Barlow *et al.,* 1982, 1984). Horseshoe crabs use visual contrast cues during mating: males can discriminate between cement castings of the female carapace and other forms. The degree of discrimination depends on the form and contrast of the castings and the time of day. Even during the new moon under starlight alone, the animals can use vision to detect high-contrast targets. It seems likely that the circadian increase of retinal sensitivity at night underlies this remarkable visual performance.

The objective of this study was to assess the day-night changes in visual performance

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under controlled laboratory conditions. We measured reflexive responses to lateral eye illumination to determine whether the probability of their occurrence follows the known circadian rhythm of retinal sensitivity. In a second experiment, we measured conditioned responses to determine whether the magnitude of the day-night change in visual performance corresponds to that of retinal sensitivity.

EXPERIMENT 1: REFLEXIVE RESPONSE TO LIGHT

Cole (1923) and Northrop and Loeb (1923) reported that *Limulus* reflexively turns toward a source of light. Both studies utilized this reflexive, visually guided behavior to test Loeb's photochemical theory of animal phototropism. Cole reported that when one lateral eye was occluded, *Limulus* constantly moved in the direction of the uncovered eye, forming circles around a light source. According to Loeb's theory, these reflexive "circus movements" represent an automatic orientation for equating the intensity of illumination on both lateral eyes. Northrop and Loeb found that *Limulus*, tethered to a corner of an aquarium by a string tied to its tail, would move toward one glass wall or the other, depending on the relative levels of illumination of light sources placed behind the walls. The animal's "automatic change in direction" was viewed as support for Loeb's phototropic theory. Although these reflexive responses of *Limulus* to light are robust, they are not well suited for our studies because the movements of the animal make it difficult to determine with precision the level of retinal illumination.

Figure 1 shows several types of reflexive light responses that can be recorded from immobilized animals. The recording of heart rate in (a) taken from an earlier study (Barlow and Palfai, 1971) shows that the onset of illumination of the lateral eyes produces a transient increase of the ongoing rate and cessation of illumination causes a transient decrease. The recording in (b) taken from the current study shows that illumination of the lateral eyes can stop gill ventilation and produce a different rate of ventilation after light offset. The electrophysiological record in (c) shows that the offset of illumination of the lateral eyes can elicit an "off" discharge in some single fibers of the abdominal nerve cord (R. Barlow and D. Goodman, unpub. obs.). As indicated in trace (d), illumination of the lateral eyes can elicit reflexive movements of the telson (tail). Tail movements also can be elicited by illumination of the ventral and median eyes (Wasserman, 1973).

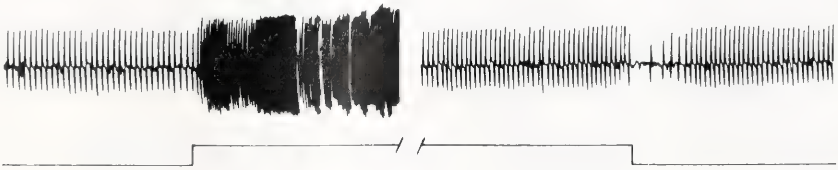
Of these four reflexive responses to light, we chose to study tail movements and gill ventilation because they are readily observed, easily recorded, and do not require surgery. Because the animal is immobile, we could also record the electroretinogram (e) simultaneously with tail movement for a convenient measure of retinal sensitivity during the reflexive response to light.

Materials and methods

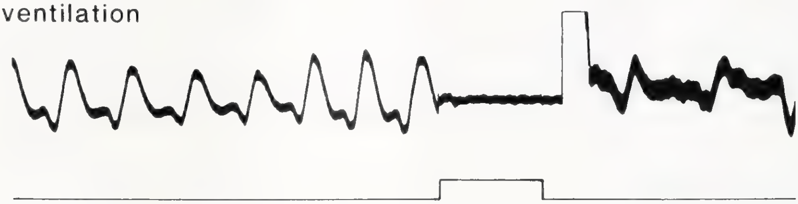
The experiment was carried out in Syracuse with adult male *Limulus* (20 to 25 cm across the carapace) shipped from the Gulf Specimen Company, Panacea, Florida. The animals were freshly caught in the spring, shipped immediately, and tested shortly after arrival in Syracuse. Animals transported to Syracuse during the summer from the Marine Biological Laboratory, Woods Hole, Massachusetts, and stored until fall or animals shipped to Syracuse during the fall did not respond.

Animals were held firmly on a platform by means of Plexiglas clamps placed around the rim of the carapace. The platform was placed in an aerated seawater aquarium such that the tail was free to move, and a bead thermistor (Model

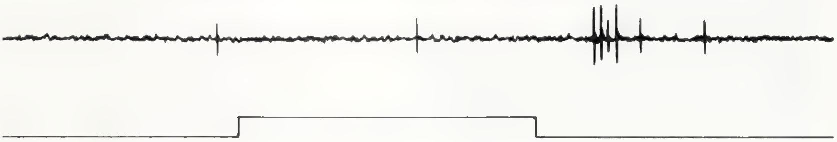
(a) Heart rate



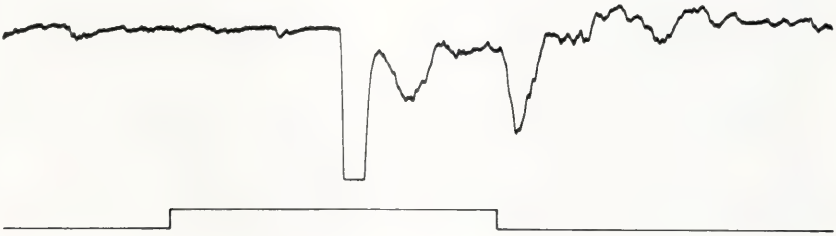
(b) Gill ventilation



(c) Abdominal nerve activity



(d) Telson movement



(e) Lateral eye ERG

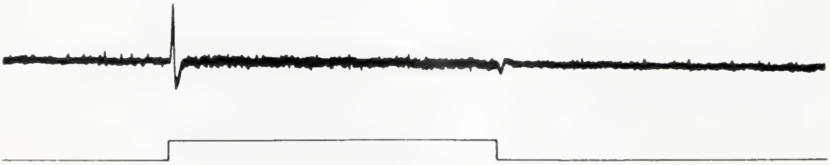


FIGURE 1. Reflexive responses to illumination of the *Limulus* lateral eye. (a) Heart rate, recorded via a pressure transducer implanted beneath the carapace (Barlow and Palfai, 1971), increased from 8 min^{-1} to more than 30 min^{-1} after light onset at 9:40 a.m. Solid line indicates retinal illumination. A transient

P60DA202M, 2K, ±20% at 25°C, Thermometrics, Edison, New York) encased in a waterproof housing (Powers and Easter, 1978) was attached to the tail with elastic bands. A small current was passed across the thermistor and the voltage drop across the device was monitored on a chart recorder. Movements of the tail momentarily cooled the thermistor, producing changes in resistance which were detected as changes in potential on the chart recorder. Complete experiments were carried out with three animals.

One lateral eye of each animal was illuminated with a light pipe (1.2 cm dia) placed 1 cm from the corneal surface parallel to the optic axis of the eye. The level of illumination was adjusted so that detectable tail movements occurred with a probablity of 0.8 for test flashes delivered during the animal's active phase. The light stimulus was a train of five monochromatic flashes ($\lambda = 520$ nm) 100 ms in duration at a rate of 5/s. Each flash delivered about 10^7 photons/cm^2 at the surface of the cornea in the experiment reported in Figure 2. The fixed intensity stimulus was presented every 15 min for four days beginning several days after the animal was placed in the dark. A "response" was defined as any detectable deflection in the thermistor record during or immediately following the train of flashes. The percent of trials that produced responses was computed every hour and averaged over the four days.

To measure retinal sensitivity, ERG responses were recorded from the test eye of the animal during the fourth day of the experiment. The technique for recording the ERG was identical to that reported previously (Barlow, 1983) except that the reference electrode was placed on the surface of the carapace near the eye instead of through a hole in the carapace. The placement of electrodes was carried out under infrared illumination. The ERG amplitude was recorded together with the tail response every 15 minutes for the next 24 hours.

Results

Figure 2 shows the amplitude of the ERG and the probability of tail movements for one animal as a function of time of day. The probability of occurrence of detectable tail responses varied from about 0.05 to 0.80 over the 24-h cycle, while the animal remained in total darkness. Tail movements were highly correlated with ERG amplitude ($r = .84$; $P < .001$), and both responses changed systematically with time of day. Other animals yielded similar results.

Note that both rhythms in Figure 2 were shifted in phase about 6 h with respect to the solar day. Previous studies of the circadian rhythm of the ERG show that maximal retinal sensitivity is centered about midnight for animals placed in darkness after exposure to solar lighting (Barlow, 1983). But for the animal in Figure 2, the period of elevated sensitivity was centered about 6 a.m., probably due to the time of day the animal was placed in a darkened container for shipping. It is interesting that both the behavioral and physiological responses exhibited the same phase shift. Apparently the rhythms of both responses are related.

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decrease in rate occurred at the offset of illumination 12 h later at 9:40 p.m. Each segment recorded in darkness is 4 min duration. (b) Gill ventilation, monitored with a thermistor (see text), is inhibited by a 5-s flash of intense light. (c) A discharge of nerve impulses is triggered in an abdominal nerve fiber at the offset of 2-s light exposure of one lateral eye. The other nerve fiber fired before, during, and after the stimulus. The activity of both fibers was recorded with a single suction electrode. (d) Telson (tail) movements, measured with a thermistor, are elicited by a 10-s flash of moderate intensity white light. (e) An ERG (120 mV peak-to-peak) was recorded from the lateral eye with a corneal electrode at the onset of the 10-s flash that elicited the tail movements in (d).

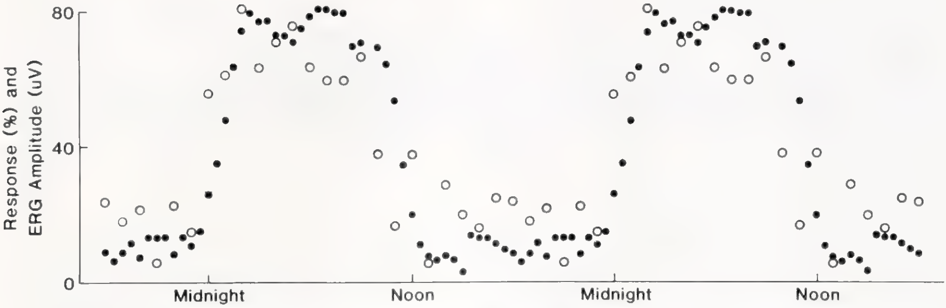


FIGURE 2. Probability of reflexive tail movements correlates with the circadian rhythm in ERG. Tail movements were elicited by constant intensity light pulses (10^7 photons/cm^2/flash) delivered every 15 min to one lateral eye while the animal remained in darkness for 4 days. Open circles give the percent of trials per hour to which the animal responded over the entire 4-day period (16 trials per point). Filled circles give the ERG amplitude recorded from the lateral eye every 15 min during the final day of the experiment. Data are double plotted.

EXPERIMENT 2: CONDITIONED RESPONSES TO LIGHT

Detailed knowledge of the physiology of the *Limulus* lateral eye from the work of Hartline and his colleagues (Hartline, 1972; Ratliff and Hartline, 1974) inspired several investigators to study behavioral responses elicited by lateral eye illumination (Smith and Baker, 1960; Makous, 1969, 1970; Wasserman and Patton, 1969, 1970; Wasserman, 1970; Barlow and Palfai, 1971). The investigators attempted to train *Limulus* to respond to light by pairing a visual stimulus with a non-visual one that alone elicited a reflexive response. These laboratory studies yielded marginal success. At best, conditioning required a large number of trials, and only a small percentage of animals were ultimately trained (Makous, 1969).

Field studies of *Limulus,* on the other hand, reveal a role for vision in the animal's mating behavior (Barlow *et al.,* 1982, 1984). Also, in Experiment 1 of this paper we found in the laboratory a correlation between the probability of reflexive visual responses and circadian changes in the sensitivity of the lateral eye. We therefore were encouraged to develop a classical conditioning paradigm for determining the magnitude of circadian changes in visual performance. Results are described here in Experiment 2. Preliminary results were presented elsewhere (Powers and Barlow, 1981).

Materials and methods

This experiment was performed during the summer at the Marine Biological Laboratory, Woods Hole, Massachusetts, where freshly collected animals are readily available. As with Experiment 1, only freshly caught animals yielded optimal results. Following the technique in Experiment 1, the animals were held firmly on a platform with a thermistor attached to the tail. Another thermistor was attached to the supporting platform beneath the gill books. A small current was passed across each thermistor, and the resulting potentials were monitored on a chart recorder. Movements of the tail and gills cooled the thermistors, changing the voltage drop across each device.

Electrical shock, the unconditioned stimulus (US), was applied to the muscle between the prosoma and opisthosoma of the carapace. Shocks, 1 ms in duration, were delivered via a Stimulus Isolation Unit driven by a Grass Stimulator (S48) at the rate of 10 pulses per s for 1 s. The voltage required to produce reliable changes in response (see *Definition of response,* below) varied from animal to animal, and sometimes from session to session; it was adjusted accordingly, but was generally between 1 V and 10 V. Figure 3A shows examples of unconditioned telson and gill responses to the presentation of shock.

Light, the conditioned stimulus (CS), was transmitted to the lateral eye by a light pipe (1.2 cm dia) placed 1 cm from the cornea. The light stimuli were 10 s in duration and their wavelength and intensity were controlled by interference and neutral density filters. The unattenuated output of the light pipe, as measured with a calibrated photodiode (PIN 10UV, United Detector Technology, Inc., Santa Monica, California) was 7.5×10^{12} photons/cm^2/s at the surface of the cornea for experiments in which an interference filter was used ($\lambda = 520$ nm), and 1.3×10^{15} photons/cm^2/s at the cornea from 400 to 650 nm for experiments using white light.

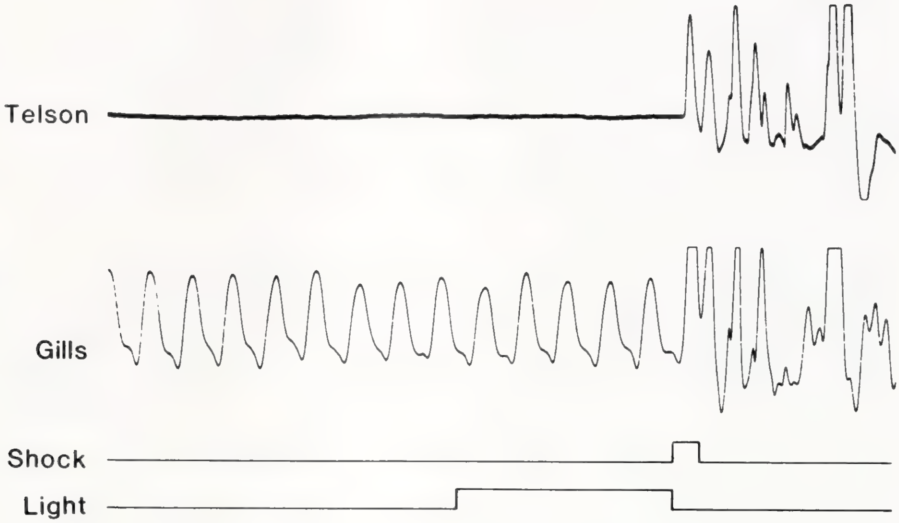
Definition of response. Following each trial, the experimenter compared the pattern and amplitude of tail and gill movements observed during the stimulus period with movements during a baseline interval prior to stimulus presentation. During habituation, training, testing, and extinction, the baseline interval was 20 s pre-CS. For trials with shock only, the baseline interval was 10 s preceding shock and the interval which was examined for response was 5 s, beginning with US presentation. We defined any observable change in either gill ventilation or tail movement or both as a response.

In an attempt to reduce the probability of being deceived by using a subjective criterion for response, each author independently scored the data from the original chart records. One of us scored responses while the experiment was in progress, and the other after its completion. We alternated in the two roles, and the person who scored the data while the experiment was in progress later coded the chart records so that the other person could rescore them without knowledge of the stage of the experiment (light intensity, training *vs.* testing, etc.).

Training and testing. Animals were positioned in the apparatus, placed in a lightproof cage, and left undisturbed in darkness for 12 to 24 hours. At the end of this period, shock amplitude was adjusted to produce reliable responses in both tail and gill movements, and training was begun. During training, the CS remained unchanged in intensity for a given animal, with a CS-US interval of 10 s and an intertrial interval of at least 15 min. For the experiments reported here, training intensities were log I = −4.9 for L9, log I = −3.7 for L10, and log I = −5.9 for L16. Training continued until 60% or greater response occurred in two successive 20-trial blocks. Training sessions consisted of 2–6 blocks, and were timed to coincide with the animal's estimated subjective night. The animal remained in the apparatus throughout training. Figure 3B shows conditioned responses to light in an animal that had received 39 previous pairings.

Once an animal was trained, its visual performance was tested in the same apparatus without removing or otherwise disturbing the animal. The test stimuli had the same chromatic composition as the training stimulus; animals trained with white light were tested with white light, and those trained with 520 nm light were tested with 520 nm light. The parameters of the US were kept constant during tests. All tests began with the intensity at which the animal had been trained and proceeded in a descending staircase as follows: if a response was detected by the experimenter, the stimulus intensity was decreased by 0.5 log unit; if there was no respone by either gills or tail, stimulus intensity was increased by 0.5 log unit. This procedure resulted in a final oscillation between stimulus values to which the animal responded regularly and those to which it did not respond. It also produced a concentration of trials at the midpoint of the psychometric function relating stimulus intensity and probability of response. Tests consisted of 25-trial blocks, with one to five additional blank trials randomly inserted during each test block. On blank trials the light source was turned off, but all

(a) Unconditioned responses



(b) Conditioned responses

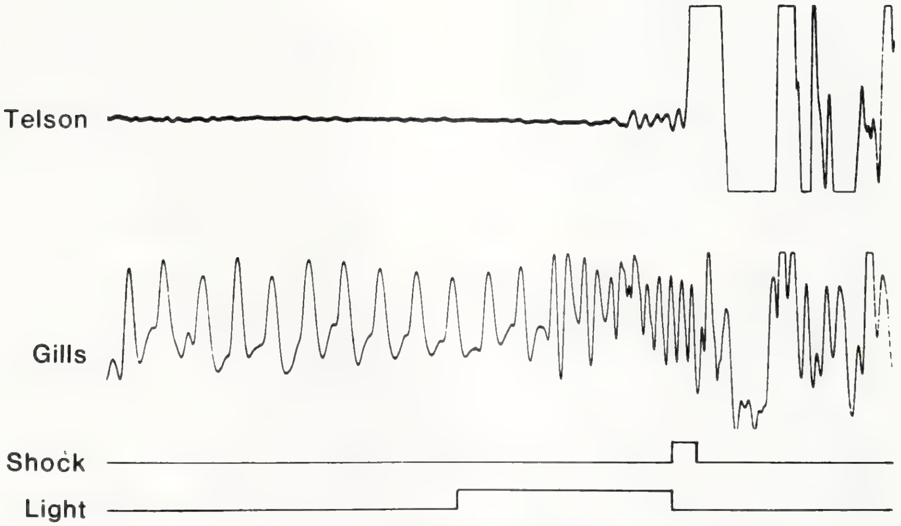


FIGURE 3. (a) Unconditioned responses of tail and gill to a 1-s series of current pulses applied near the muscle between the prosoma and opisthosoma. Neither the tail nor the gill responded to the 10-s flash of dim light (10^6 photons/cm^2/s) delivered to the lateral eye. (b) Conditioned responses of tail and gill to illumination of the lateral eye. Note responses occurred after light onset but before shock onset. This was the fourth trial of the third training session. The first session (1200–1340 h) contained 21 trials, the second (1900–2145 h) 15 trials, and the third (2345–0130 h) 14 trials.

other parameters (shutter, shock, etc.) remained the same as for a regular trial. Intertrial intervals were at least 5 min in order to maintain the lateral eye in a dark-adapted state. Animals were tested during times when retinal sensitivity is known to be maximal (subjective night) and minimal (subjective day). Most animals were tested twice during the day and twice at night, on two sequential days; some had only one test. The values reported here are the total percent response over both tests. Data from animals with >20% response to blanks were discarded.

Habituation. Most animals showed an unconditioned response to bright light (Fig. 1). To show that this response could be eliminated, several animals were exposed to the CS alone and were not exposed to the US. The CS was presented every 15 min, and responses were recorded as with conditioned animals. We measured the number of trials required for cessation of response.

Extinction. Following conditioning some animals were run in extinction sessions in which the CS alone was presented. Responses were recorded for each trial, and we counted the number of trials required for extinction.

Results

Of the 14 animals that underwent training, 9 eventually demonstrated conditioned behavior. The range of trials required to condition the nine animals was 20 to 160. The five that did not reach our criterion for training had all been collected more than seven days before training began. We attempted to measure behavioral thresholds during the day and at night for five of the conditioned animals. Below we report the threshold data for the three best cases. Data from the other two animals were similar.

Figure 4 shows representative acquisition and extinction data from one of the three animals reported below. A total of 80 CS-US paired trials were presented in four blocks of 20 trials each over a 24-h period. This animal (L16) responded on 50 to 70% of the first 40 trials, and on 80 to 90% of the last 40 trials. Testing was then carried out using the staircase method described above (see Fig. 7). Testing was followed by 40

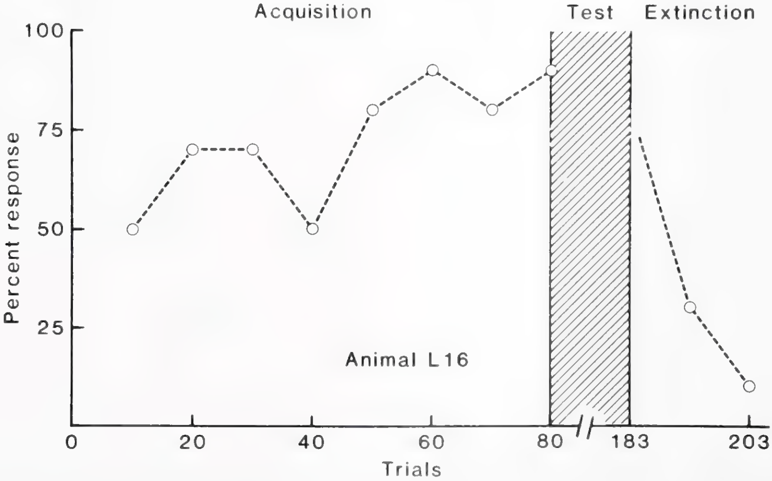


FIGURE 4. Acquisition and extinction data for animal L16. The ordinate is the percentage of trials during which a response was detected in either tail movement or gill ventilation in successive 10-trial blocks. The abscissa gives the cumulative number of trials. The conditioned stimulus delivered 7.5×10^5 photons/cm^2/s at the surface of the cornea ($\lambda = 520$ nm).

extinction trials. As Figure 4 shows, extinction of the conditioned response to light was rapid and complete after the shock was turned off.

Figure 5 shows psychometric functions for animal L9 tested with white light during the day (open circles) and at night (filled circles). It illustrates three characteristic features of *Limulus* performance during testing in the conditioning paradigm. First, the animals did not always respond to lights that were clearly above threshold (*e.g.*, from log I = −10.2 to −9.0 in Fig. 5); most conditioned animals responded to an average of only 60 to 70% of such stimuli during testing. This property did not differ between day and night conditions. Second, an intensity was nearly always found to which the animals would not respond (log I = −11.9 at night and log I = −11.5 during the day in Fig. 5). And third, intensities could be found that elicited a reliable response during the night but not during the day (*e.g.*, log I = −11.5 in Fig. 5).

The threshold of animal L9 was lower at night than during the day. We estimated the difference in sensitivity between day and night to be 0.7 log units by shifting the illustrated curves along the abscissa until the best fit was achieved by eye.

Figure 6 illustrates the inter-observer reliability of our scoring technique. The chart records for the data in Figure 5 were separately scored during the experiment by MP who knew the intensity of the stimulus and after the experiment by RB who did not. The two sets of scorings were similar with a high statistical correlation ($r = .79$, $P < .0001$ for daytime data and $r = .64$, $P < .005$ for nighttime data). The average of our two scorings was used to construct Figure 5. All other figures were constructed from scorings of one observer.

Figure 7 shows a decrease in threshold at night when conditioned animals were tested with monochromatic light ($\lambda = 520$ nm). This figure combines results from two animals. Note that the data from one animal resemble those from the other on an

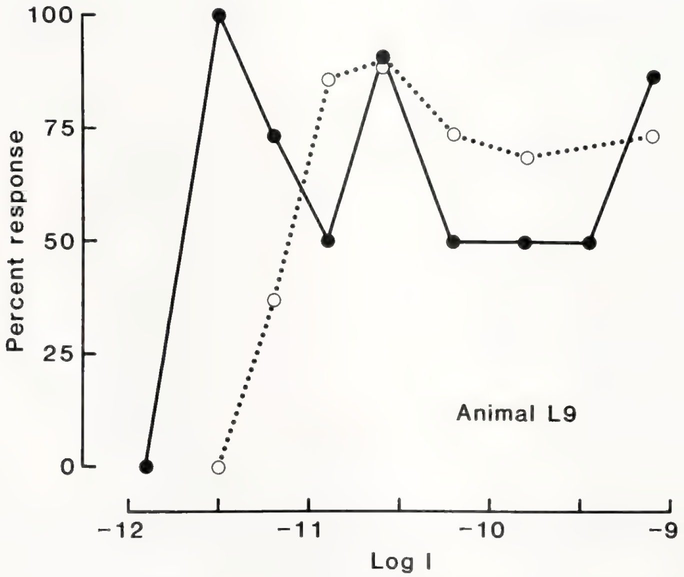


FIGURE 5. Psychometric functions for animal L9 conditioned to respond to flashes of white light. Percentage of trials containing detectable responses of either the tail or gill during the day (open circles) and night (filled circles) is plotted on the ordinate as a function of the relative intensity of the conditioned stimulus plotted on the abscissa. Each point is the average of the two values plotted in Figure 6.

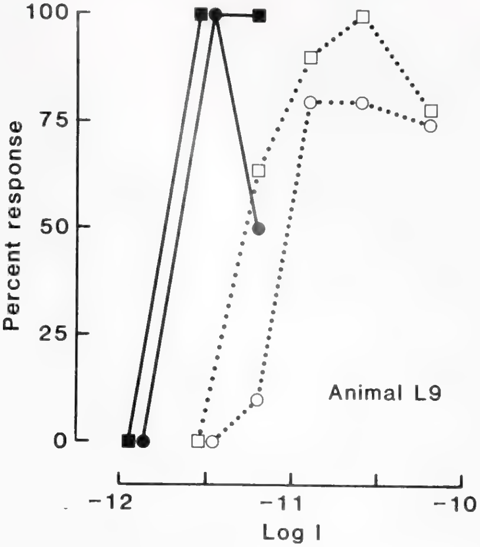


FIGURE 6. Individual scoring by the two authors of part of the data in Figure 5. MP (squares) judged whether responses had occurred during each trial while the experiment was in progress. RB (circles) scored the same data after completion of the experiment with no information about stimulus intensity. Open symbols are daytime data and filled symbols are nighttime data. Axes are the same as in Figure 5.

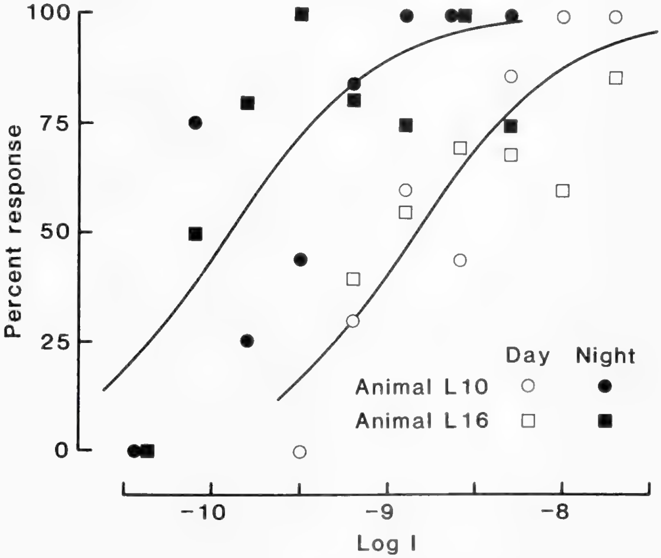


FIGURE 7. Percent response as a function of light intensity for two animals conditioned to respond to flashes of monochromatic light ($\lambda = 520$ nm). Daytime (open symbols) and nighttime (filled symbols) results are given for male animals L10 (circles) and L16 (squares). Axes are the same as in Figure 5. The intensity at the surface of the cornea was 750 photons/cm^2/s at log I = -10. Curves are hyperbolic tangent functions fitted to the average data for both animals by least-squares regression analysis. The difference between the curves at 60% response is 1.1 log units, showing that on average animals were 13 times more sensitive at night than during the day.

absolute scale, and that the features present in Figure 5 are also present here: some intensities elicited no responses and some that elicited responses at night elicited no response during the day.

The average decrease in threshold at night was determined by fitting hyperbolic tangent functions (solid lines) to the combined sets of data. The fitted curves are separated by 1.1 log units, indicating that on the average the animals were about 13 times more sensitive at night than during the day. Fitting hyperbolic tangent functions to the individual data (curves not shown) yielded nighttime increases in visual sensitivity of 0.92 log units for animal L10 and 1.27 log units for animal L16. The mean day-night difference in sensitivity for all three animals (L9, L10, and L16) was 1.0 log unit.

DISCUSSION

The visual sensitivity of *Limulus* measured behaviorally changes with time of day. Animals kept in constant darkness exhibited higher sensitivity at night than during the day. The changes in sensitivity are similar in time course (Experiment 1) and in magnitude (Experiment 2) to the circadian rhythms in retinal sensitivity recorded physiologically (Barlow, 1983, see below). Thus it seems reasonable to conclude that the changes in visual sensitivity measured behaviorally result from rhythmic changes in retinal sensitivity generated by a circadian clock. Although the clock is located in the protocerebrum of the central nervous system (Eisele *et al.*, 1982), its known influence on the lateral eye is sufficient to account for the changes in behavior reported here.

Endogenous changes in visual sensitivity were detected for both conditioned and unconditioned responses; however, the latter required substantially higher levels of retinal illumination. For example, to elicit unconditioned tail movements with high probability (>.60) at night required light flashes delivering 10^8 photons/cm^2/s at the surface of the cornea (Fig. 2). On the other hand, conditioned tail responses were evoked at the same rate at night by light flashes containing 2.5×10^3 photons/cm^2/s (Fig. 5). The difference probably reflects the increased arousal level of the animal in the conditioning experiment due to the association of shock with the visual stimulus.

Comparison of behavioral and physiological results

Physiological recordings indicate that less than 10 photons/s incident on a single ommatidium are sufficient to elicit an optic nerve discharge at night (Barlow *et al.*, 1977). As mentioned above, flashes containing 2.5×10^3 photons/cm^2/s ($\log I = -11.7$ in Fig. 5) were required at night to elicit reliable conditioned tail or gill movements. The adult retina is about 0.8 cm^2 in area and contains about 900 ommatidia, giving a receptor density of approximately 1100 ommatidia/cm^2. Thus the nighttime threshold in Figure 5 is equivalent to about 2.3 photons/s incident on a single ommatidium. This threshold flux corresponds well to that measured physiologically at night.

The daytime threshold in Figure 5 is 1.3×10^4 photons/cm^2/s ($\log I = -11.0$ at 60% response) which is equivalent to 12 photons/s incident in a single ommatidium. This threshold flux is much lower than the physiologically measured value of 1000 photons/s/ommatidium (Barlow *et al.*, 1977). One possible explanation for the discrepancy is that the physiological threshold is based on the response of a single optic nerve fiber, whereas the behavioral threshold results from the combined activities of all optic nerve fibers. During the day the high level of spontaneous activity of a single optic nerve fiber in the dark (1–5 impulses/s) can mask responses of a single receptor to low levels of illumination (Barlow *et al.*, 1977). If the spontaneous activity of each optic nerve fiber is independent of that of the others, then summing across an ensemble of n nerve fibers can reduce threshold by a factor of $n^{-1/2}$ for large field stimuli. In the behavioral experiments, light stimuli illuminated the entire array of approximately 900 ommatidia in the adult eye. Since each ommatidium transmits nerve impulses to the brain over a single nerve fiber, the intensities required to elicit detectable responses in the behavioral experiments may be about 30 times lower than those required for single fiber responses. Thus the physiological threshold of 1000 photons/s/ommatidium for single receptor illumination would be reduced to about 30 photons/s/ommatidium for whole eye illumination. This lower value is within range of the behavioral threshold of 12 photons/s/ommatidium in Figure 5.

Visual sensitivity measured behaviorally during the day therefore agrees reasonably well with that measured physiologically if the intrinsic noise of the retina is considered. At night behavioral and physiological measures of sensitivity agree well without consideration of optic nerve noise, possibly because such noise is suppressed by a circadian clock located in the brain of *Limulus.* We measured the nighttime behavioral thresholds during the period (9 p.m. to midnight) when efferent optic nerve activity generated by the clock is known to nearly abolish the spontaneous afferent optic nerve activity generated by the retina (Barlow *et al.*, 1977).

Laboratory vs. *sea conditions*

All of our behavioral measurements were carried out in the laboratory with animals maintained under dark adapted conditions so we could investigate the endogenous changes in visual sensitivity. In the animal's natural habitat, the sea, daily fluctuations in ambient illumination produce additional changes in visual sensitivity. Light adaptation reduces visual sensitivity and abolishes spontaneous activity in optic nerve fibers (Kaplan and Barlow, 1975). As a consequence, the masking effects of retinal noise discussed above need not be considered when the animal is light adapted. It appears likely that under natural conditions the visual performance of the animal is probably not impaired at any time by retinal noise which is reduced by light adaptation during the day and by a clock at night.

Behavioral manifestations of circadian rhythms in retinal sensitivity are rarely measured; instead, physiological studies have been the rule. One reason is the difficulty of reliably eliciting and observing behaviors for the long periods necessary to demonstrate all characteristics of circadian rhythms. Partly because of such limitations, the evidence for circadian changes in sensitivity reported here is indirect. Nonetheless, our findings that (1) the probability of eliciting a particular visually mediated behavior correlates highly with the circadian changes in the ERG, and (2) the changes in absolute sensitivity correspond with those measured physiologically, strongly suggest that these visually evoked behaviors are governed by the same mechanism (clock) that generates the circadian changes in retinal physiology. It is interesting to note that visual sensitivity of humans (Bassi and Powers, submitted) and rats (Rosenswasser *et al.*, 1979) measured behaviorally also appears to vary on a circadian cycle.

True conditioning vs. *pseudoconditioning*

Demonstrating true conditioned responses in *Limulus* has been problematic, and the results reported here are no exception. First we, like Cole (1924), observed that *Limulus* maintained in captivity for more than about a week, do not perform well in behavioral tasks. In our hands, only those animals that had been in the lab for less than a week at the time the experiment began gave reliable responses. Second, previous experiments on conditioning in *Limulus* have been criticized for not including control procedures for pseudoconditioning (Makous, 1969). We also have not done so in this study, but preliminary experiments using similar procedures showed that pseudoconditioning did not occur (R. Barlow, unpub. data). Whether animals were truly conditioned in the present study seems to us to be of less importance, however, than the demonstration that changes in the relative and absolute visual sensitivity of the animal can be predicted by the endogenous physiological changes of its visual system.

Possible role of circadian changes in visual sensitivity for Limulus *in its natural habitat*

Animals in the population from which our sample was drawn mate during late spring and early summer, predominantly during the high tide at night. The relative phase of the lunar and solar days and the level of ambient illumination appear to play critical roles in the animal's mating behavior (Howard *et al.,* 1984). However, males in search of mates can visually detect a female-like object under water regardless of the time of day (Barlow *et al.,* 1984). Such excellent visual performance in the presence of large fluctuations in ambient illumination is remarkable in a species that possesses only one receptor type. We speculate that the circadian changes in retinal sensitivity help both male and female *Limulus* determine whether the environmental conditions are appropriate for mating and, if so, help males locate and approach females during day and night.

ACKNOWLEDGMENTS

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ALTERNATIVE MATING STRATEGIES IN MALE MORPHOTYPES OF THE FRESHWATER PRAWN *MACROBRACHIUM ROSENBERGII* (DE MAN)

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ABSTRACT

Males in a mature, single-age, pond population of the freshwater prawn *Macrobrachium rosenbergii,* can be divided into three distinct morphological types, representing three phases in the male developmental pathway (Brody *et al.,* 1980). Behavioral and physical characteristics of all three morphotypes were examined with regard to mating behavior and reproductive probabilities.

Two alternative mating strategies are described. The largest, dominant males actively court and protect the females prior to mating. Males of the intermediate category demonstrate a reduced rate of reproductive activities in the presence of dominant males. The smallest males practice a form of sneak mating consistent with their small size and high mobility. A cost-benefit balance for each particular strategy is offered, based on qualitative features such as morphology and behavioral observations, as well as on quantitative data, including growth rates, relative proportion of each morphotype in the male population, and the respective frequencies of social encounters with females, which result in successful matings, or, in fatal injuries.

INTRODUCTION

A social organization with obvious size dependent hierarchical characteristics and aggressive tendencies at both the alimentary and breeding levels is known to exist in a number of species of insects (Alcock *et al.,* 1977; Ward, 1983) and in aquatic organisms (Collins *et al.,* 1967; Gandolfi, 1971; Constantz, 1975; Dominey, 1980). When male reproductive success depends on male-male competition and aggression, as is usually the case in polygamous species, individuals which are at a competitive disadvantage sometimes adopt an entirely different constellation of reproductive behaviors. In most cases, individuals practice only a single reproduction option throughout their lifetime. However, when such alternative mating patterns are practiced as part of a developmental sequence, they can be considered parts of a single lifetime reproductive strategy (Dominey, 1980).

The reproduction behavior of the freshwater prawn *Macrobrachium rosenbergii* falls into the latter category. A single age population is characterized by a sex-associated size distribution in which the weights of females are quite homogeneous, while the males, at nearly 1:1 ratio with the females, yield individual weights to form a wide, positively skewed distribution, with half the population being considerably larger than the females, and half being quite small (Smith *et al.,* 1978; Ra'anan and Cohen, 1985). A close examination of the males reveals three distinct morphotypes based upon size

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ranking, claw color, and the ratio of claw length to body length (relative claw length). The three male categories include (a) small (SM) males, represented in the peak of the male distribution, characterized by clear chelae with a relative length of 0.5–0.7; (b) orange claw (OC) males, larger than SM males, characterized by the orange color of their chelae and by a relative claw length of 1.0–1.5; and (c) blue claw (BC) males, the largest individuals of the male population, characterized by thick dark blue chelae with a relative claw length of 1.5–2.0. Whereas the weight of small males is mainly restricted to the range between 1–10 g, the weight and size of OC and BC males vary widely with cultural procedures. Nevertheless, the relative proportions of the three male morphotypes, SM, OC, and BC, remain nearly constant at 5:4:1, respectively, under a wide range of environmental conditions (Brody *et al.*, 1980; Cohen *et al.*, 1981). These ratios are maintained at a dynamic state, in which individual males are capable of undergoing a transformation from one morphotype to another, following an irreversible order: from SM to OC to BC. Such transformations would occur whenever large individuals either die or are selectively removed (Ra'anan and Cohen, 1985).

Aquarium and field observations on the three male morphotypes suggested the existence of a reproductive-dominance hierarchy in which the BC males are the climax of the male development pathway. A BC male is dominant, territorial, and sexually active, usually in association with eight to ten females (Raman, 1967). Whenever a female becomes receptive to fertilization, the BC male displays a characteristic courtship behavior which is followed by mating. OC males are sub-dominant, non-territorial, and were never observed courting and protecting a receptive female, although occasional fertilizations by OC males have been recorded (Ra'anan and Cohen, 1985). Small males are not territorial, they are submissive to all other males and are highly mobile (Peebles, 1980; Harpaz, 1980). SM males were observed to be strongly attracted to receptive females, and in several events, females were successfully fertilized when only SM males were present, indicating the SM males are sexually competent (Sagi, 1984).

The present study evaluates the relative reproductive potential of each of the morphotypes while examining their mating strategy by means of aquarium observations. We have also tried to arrive at a qualitative cost-benefit evaluation of each of these morphotypes (representing three different developmental stages) according to each type's specific physical characteristics, typical behavior, and probability of achieving successful fertilization.

MATERIALS AND METHODS

Source of animals

Prawns for this study were obtained from a commercial fish pond stocked with a single age juvenile population, so that all individuals were of the same hatching batch and were reared under the same environmental conditions.

The selection of males was performed according to the morphologically distinctive characteristics of the three male categories, and the selection of females was based on their state of sexual maturation as determined by gonad development (Sagi and Ra'anan, 1985).

Experimental groups

Mating probability of SM and OC males was examined in aquaria by stocking each of these morphotypes either alone with females or together with a dominant BC male. In addition, the dominant BC male, in one experimental group, was operated on so that it was unable to release its own spermatophores. This was performed by sealing the gonopores with a drop of quick-set adhesive cyanoacrilate (commercial name: Super Glue). We have previously shown that such treatment does not interfere with the male's display of courting behavior (Sagi and Ra'anan, 1985). The rate of female fertilization by the subordinate SM and OC males in the presence of a 'blocked' BC male indicates the actual probability of SM and OC males to achieve a successful copulation in a situation more similar to that prevailing in nature where BC males are guarding and protecting receptive females.

The number of males distributed to the aquaria was chosen to simulate the morphotypes' relative frequency within the male population in nature (Cohen *et al.,* 1981), and was limited by the size of the aquarium. The following stocking combinations were set up:

(a) 6 SM males (5–10 g each)
(b) 3 OC males (30–40 g each)
(c) 3 BC males (40–50 g each)
(d) 1 BC male and 5 SM males
(e) 1 BC male and 2 OC males
(f) 1 blocked BC male and 5 SM males
(g) 1 blocked BC male and 2 OC males

Three aquaria were set up for each stocking combination. Whenever a male went through a morphotypic transformation, died, or suffered a physical injury (such as lost claws while molting or fighting), it was replaced by another male of a similar morphotype. Altogether, all males in each aquarium were replaced 5–6 times within the entire observation period of 5 months.

In addition to the males, a female with ripe gonads was present at any given time in each aquarium. Whenever a female became berried, it was replaced by an equivalent female, and was transferred into a separate aquarium for a 15 day follow-up of embryonic development. When a receptive female in an observation aquarium either did not become berried, or lost all eggs within 48 hours from the pre-mating molt, it was replaced by another female and a failure of fertilization was recorded. Altogether, 16–45 females were examined for each of the male stocking combinations.

Experimental conditions

Observations were carried out in 130 l glass aquaria (100 × 37 × 39 cm), equipped with built-in biofilters and temperature control devices which kept a constant temperature of 25–27°C. Each aquarium contained substrates in the form of six or seven plastic pipes, 10 cm in diameter and 20–25 cm long, which provided shelters for newly molted individuals.

All animals were fed daily with minced fish fillet and live *Daphnia,* occasionally supplemented with commercial fish pellets (25% protein). Uneaten food and other accumulated particulate matter were removed from each aquaria by siphoning once a week.

Aquaria were checked daily for molts, male morphotypic transformations, and berried females.

Berried females were kept isolated in 8 l aquaria (20 × 20 × 40 cm), also equipped with biofilters and kept under the same temperature.

Observations and measurements

Behavioral observations were carried out for a few hours at a time during daytime only, especially when a pre-mating female was observed. Special attention was given to the relative position of each individual in the aquarium, to the typical male-male interactions (agonistic behavior) and to the male-female interactions (protecting and mating).

Whenever a female was removed, some 48 hours after a pre-mating molt, its physical state was recorded, *i.e.*, lost appendages, broken antennae, and other signs of physical injury. Embryonic development was determined two weeks later by a microscopical examination of eggs sampled from the female's abdomen and served as the ultimate proof of successful fertilization.

The percentages of successful fertilizations, failures to fertilize, female mortalities due to physical injuries inflicted by males soon after pre-mating molt, and other female mortalities unrelated to interactions with males, were calculated. Differences in the relative proportions of each of these categories among the various male stocking combinations were tested by a χ^2 contingency table.

RESULTS

Behavioral characteristics of the three male morphotypes

A qualitative comparison of the mating strategies of the three male morphotypes is presented in Table I. This table shows the main characteristic features of each morphotype and the typical behavior observed when a receptive female is present.

A major behavioral difference is apparent between BC and SM males. BC males spend most of the time resting and grooming themselves, or else they are actively courting and protecting the receptive female while displaying agonistic behavior toward all other approaching individuals, males and females.

SM males are highly mobile. They are the first to react to the introduction of food and are strongly attracted to receptive females. They usually avoid physical contact with superior males, both BC and OC males, by retreating into the water column whenever closely threatened. However, there are always a few SM males continuously hovering around a female, whether the female is alone or protected by a BC male. Occasionally, an SM male would attempt to sneak under the female and to attach its spermatophore to the female's abdomen. This mating strategy may be referred to as 'sneak copulation.' Being quick and small enough, SM males were observed sneaking in between the BC male and the female as is shown in Figure 1. In the absence of a

TABLE I

Qualitative comparison of the main characteristics, physical and behavioral, of the three male morphotypes

Category	BC	OC	SM
a) Body size	Large	Large	Small
b) Relative claw length	1.5–2.0	1.0–1.5	0.5–0.7
c) Claw color	Dark blue	Orange	Clear or pink
d) Mobility	Mainly resting and self-grooming	Mainly resting and self-grooming	Mainly exploring
e) Agonistic competition	Aggressive and dominant	Aggressive but subdominant	Submissive
f) Courtship	Protecting and grooming	Never observed courting	Never observed courting
g) Copulatory attempts	Must turn over	Must turn over	Sneaking while turning itself underneath

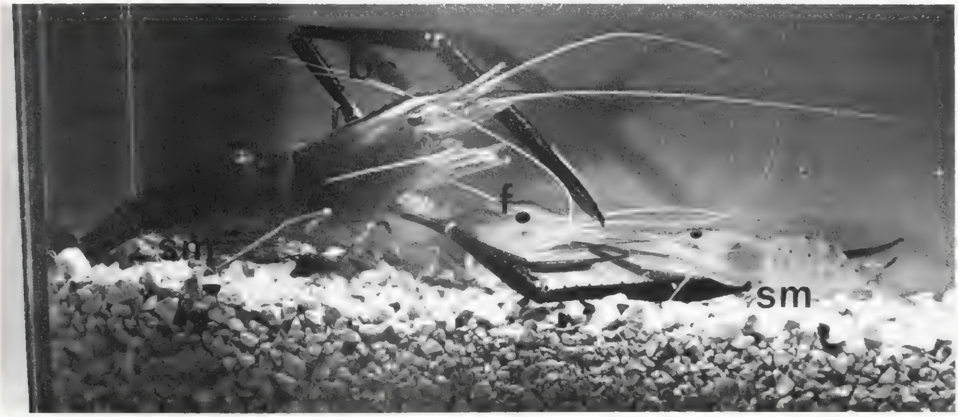

FIGURE 1. Small males (sm) in a sneaking attempt between a dominant blue claw (bc) male and a female (f).

BC male, a female would occasionally be injured by the continuing attempts of several SM males to approach her at the same time.

OC males are almost as immobile as the territorial BC males. Although they were attracted to receptive females, they were easily chased away by the dominant BC male when present, and in many instances after one or two agonistic encounters they would ignore the mating couple. In the absence of a BC male, a series of agonistic encounters among the OC males would result in one of them (usually the largest) trying to capture the female prior to mating. While receptive females were observed actively seeking the protection of BC males, they tried to avoid both SM and OC males. Whereas a SM male can steal under the female for copulation, the large OC and BC males must turn the female over, and actual copulation may occur only when the female's abdomen is exposed to an upright position. OC males were never observed protecting or grooming a female prior to mating (as is the normal courting behavior of BC males), and, indeed, in many instances a female would be badly, sometimes fatally, hurt by an OC male.

Reproductive potential of the three male morphotypes when separated

A comparison between the relative frequencies of successful fertilizations by the three male morphotypes (Table II, treatments 1, 2, 3) clearly shows the reproductive advantage of BC males over SM and OC males. While 93.3% of the females were successfully fertilized in the presence of a BC male and only 3.3% failed to become berried, in the cases of SM and OC males, successful fertilization occurred in 33.3% and 37.5% of the females, respectively, while infertility was recorded for 42.2% and 43.6% of the females, respectively. The reproductive success of the BC male was significantly higher than that of the two other morphotypes ($P < 0.05$) which did not differ significantly between themselves.

In addition to the high probability of achieving a successful fertilization, BC proved to be better guards of receptive newly molted females than SM and OC males. Only BC males were observed to protect the females during courtship activity. Moreover, only one female (2.2%) died as a consequence of male aggression whenever BC males were present, whereas females stocked with SM and OC males, had a mortality rate of 22.2% and 18.7%, respectively.

TABLE II

Reproduction potential of the three male morphotypes when held separately and in combinations with a female*

Treatment #	Stocking combination	Successful fertilization**	Failure to fertilize	Mortality due to inflicted injuries	Natural mortality
1	3 BC	93.3% (a) (28)	3.3% (a) (1)	0 (a)	3.3% (1)
2	3 OC	37.5% (b) (6)	43.6% (b) (7)	18.7% (b) (3)	0
3	6 SM	33.3% (b) (15)	42.2% (b) (19)	22.2% (b) (10)	2.2% (1)
4	1 blocked BC + 2 OC	16.6% (c) (3)	77.7% (c) (14)	0 (a)	5.5% (1)
5	1 BC + 2 OC	100.0% (a) (19)	0 (a)	0 (a)	0
6	1 blocked BC + 5 SM	33.3% (b) (15)	51.1% (d) (23)	2.2% (a) (1)	13.3% (6)
7	1 BC + 5 SM	84.4% (a) (38)	4.4% (a) (2)	0 (a)	11.2% (5)

* Index letters indicate statistical significance of differences between stocking combinations within each column. The values bearing the same index letter within each column do not show a significant difference at the level of $\alpha = 0.05$ (χ^2 contingency table).

** Numbers in parentheses indicate # of individuals.

Reproductive potential of SM and OC males in the presence of a dominant BC male

Stocking of OC males with a blocked BC male (Table II, treatment 4) ensures that berried females were fertilized exclusively by OC males. The frequency of successful fertilizations by OC males differed significantly, 37.5% *versus* 16.6% ($P < 0.05$), when held by themselves or in the presence of a blocked BC male, respectively. Failures of OC males to fertilize were recorded in 77.7% of the cases when a BC male was present, as compared with 43.6% when OC males were kept alone. In short, BC males reduced the chances of fertilization by OC males.

The reproduction potential of SM males, in contrast to that of OC males, was not affected by the presence of a blocked BC male. Thirty-three percent of the females were fertilized whether SM males were held separately or were kept with a BC male, indicating that the chances for a successful mating of SM males, applying the 'sneak copulation' strategy, are independent of the presence of dominant BC males.

Although blocked BC males could not achieve fertilization, they could still pursue their normal courting and protecting behavior, as indicated by the negligible percentage of females which died of physical injuries in their presence (0 and 2.2% in treatments 4 and 6, respectively).

In the control treatments, where SM or OC males were kept together with untreated, normal BC males (Table II, treatments 5 and 7), 84.4% and 100% of the females were successfully fertilized, indicating that females may readily become berried, under these experimental conditions, depending only on the ability of the male morphotypes present, to complete successful matings.

DISCUSSION

Blue claw (BC) males and small (SM) males may represent two alternative ways of mating while the orange claw (OC) males may be considered an intermediate developmental stage between the two, with a relatively low mating probability as such, in the presence of a BC male. This notion is supported by the fact that both SM and BC males hardly increase in body size while OC males are characterized by a rapid growth rate (Smith *et al.*, 1979; Ra'anan and Cohen, 1985), thus, investing relatively little energy, if any, in reproduction during this stage of development. The BC mating strategy may rely on superiority in threatening display towards the other males, while investing a great deal of energy in developing its weaponry (large, massive claws), defending a territory, and protecting the females. The SM male strategy, designated as 'sneak copulation,' takes advantage of the male's small body size and high mobility. This form is inferior in physical encounters, but wastes little energy in generating fighting devices.

Table III summarizes most of the apparent costs and benefits for the two extreme male developmental stages. The information gathered in this table is based on the present study (Table I, II), personal qualitative observations, and literature sources. In general, BC males seem to enjoy most of the benefits which contribute to male reproduction success, namely, high attractiveness for females, advantage in agonistic encounters with other males, and high survival probability of fertilized females following mating. These benefits are expressed in the high fertilization rate observed whenever a normally functioning BC male was present (85–100%). The main costs associated with BC mating strategy involve energy expenditure in defending a territory, reduced flexibility to readjust to changing environmental conditions, and the reduced ability to molt and thereby regenerate lost limbs, resulting in a relatively short life span. SM males, on the other hand, are less attractive to females. However, their ability to readjust quickly to changing conditions, together with the ever-present option to shift to the OC rapid growing phase, and its associated chance of becoming a BC male, may balance the lower probability of fertilizing females while being a SM male (only 33% under the present experimental conditions).

Polymorphism among sexually mature males is known for some insect species (Wilson, 1971; Alcock *et al.*, 1977; Ward, 1983) and mainly for vertebrates (Keenleyside, 1972; Constantz, 1975; Brown, 1975). In some cases, the alternative solutions for mating were assumed to be genetically based. A mechanism which would continually act toward an equalization of selective advantages of two alternatives and would therefore lead to a persistent dimorphism was offered by Gadgil (1972). Also relevant in this regard, a sex-linked gene in the platy fish, *Xiphophorus maculatus* (Kallman *et al.*, 1973), has been shown to control the age and thereby the size at which maturation of the gonadotrophic zone occurs. For other similar situations, in which large territorial males and small 'sneak' males coexist in a population (the megachilid bee *Antidium manicatum* L., and the anthophorid bee *Centis pallida*, Alcock *et al.*, 1977), it was suggested that the male polymorphism is maintained by means of parental manipulation, *e.g.*, the way in which a female divides her time and energy among her male progeny.

In the case of *M. rosenbergii*, neither genetic differences nor parental manipulation can account directly for male polymorphism. The former possibility is unlikely since we have proven that a SM male is capable of transforming into an OC male and eventually becoming a dominant BC male when the number of BC males in the population is reduced (Cohen *et al.*, 1981). It is possible, however, that some differences in genes which direct the individual relative growth rate, determine the preliminary size hierarchy observed already in early juvenile stages (Ra'anan and Cohen, 1984a, b). From that stage on, the order in which male transformation occurs might be dictated by size ranking. The second possibility of parental manipulation does not apply to

TABLE III

Costs and benefits of contrasting mating strategies of the two extreme developmental stages (SM and BC) in M. rosenbergii *male developmental pathway*

	Dominant BC males		Submissive SM	
Categories	Cost	Benefit	Cost	Benefit
a) Body size	Reduced sexual activity during OC phase	Higher competitive ability	Lower competitive ability	Less time to sexual maturity, small enough to sneak
b) Claw length and color	High investment in weaponry; easier predator target	Increased threatening effect on males: increased attractiveness to females	Disadvantage in agonistic encounters	Little energy invested in fighting devices; less conspicuous to predators
c) Mobility	Reduced ability to adapt to changing environmental conditions (personal field observations; Peebles, 1979)	Little energy expenditure	Energy investment in high mobility	Quick enough for sneak copulation; able to move to better areas when local conditions worsen (Peebles, 1979)
d) Courtship and copulatory attempts	Time and energy consuming	Increased probability of female survival after mating	Inflicting physical injuries to females, risking female mortality; high energy investment in hovering and sneaking	
e) Somatic growth and molting frequency	Reduced ability to regenerate lost limbs; physical deterioration due to lack of molting (Smith *et al.*, 1979; Peebles, 1979); low longevity (Ra'anan and Cohen, 1985)	No energy invested in growing or molting		Quick regeneration of lost limbs; high longevity
f) Male morphotypic transformation	Irreversible (Ra'anan, 1982)	Top status	Lowest status	Capable of becoming OC and eventually BC males (Ra'anan, 1982; Ra'anan & Cohen, 1985)

M. rosenbergii since there is no parental care after the eggs hatch, and the size distribution of the eggs is normal (Ra'anan, 1982).

We suggest that the male's mating strategy in *M. rosenbergii* is dependent primarily on the individual's size ranking within the population. A direct relationship between relative size and mating behavior was also described in the cases of the dung flies *Sepsis cynipsea* (Ward, 1983), and *Scatophaga stercoraria* (Borgia, 1981). In both cases most of the size variation was attributed to environmental causes. Constantz (1975) suggested that a highly competitive environment, in which there is a high frequency of large males, may favor small, sneaking males, since territorial males primarily will be occupied with agonistic behavior that consumes a significant fraction of their time and energy. By contrast, if the frequency of large males is low, their relative reproduction advantage over the small males should increase. At an evolutionary stable strategy, the relative proportions of the two male types in the population should be at an equilibrium in which the fitness of both is equal (Maynard Smith and Price, 1973). This hypothesis is consistent with the existing observations of a constant frequency distribution of the three male morphotypes of *M. rosenbergii* over a wide range of population densities and ecological conditions in the ponds (Cohen *et al.*, 1981). The signals by which any individual determines its relative position within the size hierarchy, and the mechanisms by which these signals are translated to mating behavior and to the regulation of growth rate are presently unknown.

ACKNOWLEDGMENTS

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SPERM TRANSFER AND STORAGE IN THE BROODING BIVALVE *MYSELLA TUMIDA*

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ABSTRACT

Mysella tumida has a seasonal reproductive cycle at Patricia Bay, Vancouver Island, Canada. The onset of brooding in the population is preceded by a period of exogonadal sperm storage, which persists for 1–4 months. Sperm storage is achieved by the mass attachment of spermatozoa to the abfrontal unciliated surface of gill filaments in the ascending lamellae, by means of fine microvilli which radiate from the acrosomal end of the sperm heads and interdigitate with the gill epithelial cell microvilli. Eggs are spawned into the gill chamber and are fertilized by the stored spermatozoa. Sperm transfer between individuals involves the production, release, and uptake of spermatophores. Spermatophores are released from and gain re-entry to the suprabranchial chamber through the exhalent opening. This method of sperm transfer and storage results in a high fertilization efficiency; *e.g.*, 99.9% of 39,660 eggs spawned by 50 individuals examined were fertilized. Available data indicate that *M. tumida* normally outcrosses, but the possibility of facultative selfing is not excluded.

INTRODUCTION

The majority of bivalve mollusc species are gonochoric broadcast spawners which undergo external fertilization (Sastry, 1979). The smaller members of many bivalve families, however, exhibit some form of brood protection (Sellmer, 1967; Sastry, 1979), a trait typically associated with hermaphroditism (Strathmann *et al.*, 1984). All members of the superfamily Galeommatacea investigated to date, brood their embryos in the suprabranchial chamber (Sellmer, 1967; Sastry, 1979), with the exception of *Entovalva mirabilis* and *Montacuta percompressa* which also utilize the general mantle cavity as a brood chamber (Voeltzkow, 1891; Chanley and Chanley, 1970). Fertilization occurs within the brood chamber, although the method by which the gametes are brought together to achieve fertilization has not been demonstrated for most galeommatacean species.

Oldfield (1964) and Sellmer (1967) speculate that in outcrossing species of brooding bivalves, spermatozoa are released into the surrounding water by one individual and are entrained by the inhalent current of a recipient animal into the brood chamber where they fertilize the eggs. This may explain how fertilization occurs in mantle cavity brooders, such as *Montacuta percompressa* and *Entovalva mirabilis,* but leaves unresolved the question of how sperm of an outcrossing ctenidial brooder gain access to the eggs. Galeommatacean gills are of the eulamellibranchiate type, which have been shown to filter out particles <4 μm in diameter from the water entering the suprabranchial chamber (Mohlenberg *et al.*, 1978). Sperm placed in the inhalent current of the ctenidial brooder *Mysella bidentata* did not enter the suprabranchial chamber and were frequently passed to the animal's mouth and ingested (Ockelmann and Muus, 1978).

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A number of brooding bivalves exhibit some form of sperm storage. In *Montacuta substriata, M. ferruginosa, Pythinella cuneata,* and *Entovalva perrieri*, sperm masses enclosed in membranous envelopes have been observed in the suprabranchial chambers of adults (Oldfield, 1964; Gage, 1968; Ó Foighil, 1985a). Morton describes the occurrence of sperm morulae in the gill chamber of *Pseudopythina subsinuata* (1972) and *Gaimardia finlayi* (1979). An alternate method of sperm storage which involves the en masse attachment of sperm cells to specialized regions of the gill suspensory membranes and gill lamellae occurs in *Xylophaga dorsalis* (Purchon, 1941), *Mysella bidentata* (Deroux, 1961; Ockelmann and Muus, 1978) and *Montacutona compacta* (Morton, 1980). These sperm storing regions have been interpreted as seminal receptacles (Purchon, 1941; Ockelmann and Muus, 1978; Morton, 1980) and the stored sperm are characteristically orientated with their heads pointed towards the receptacle epithelium. The method of sperm-epithelium attachment is unknown.

Species of *Mysella* are hermaphroditic and brood embryos in the suprabranchial chamber to a straight-hinged veliger stage (Lovén, 1848; Lebour, 1938; Miyazaki, 1936; Franz, 1973; Ockelmann and Muus, 1978; Ó Foighil *et al.*, 1984). The complicated reproductive cycle of the North Eastern Atlantic species *Mysella bidentata* has been investigated in detail by Deroux (1961) and Ockelmann and Muus (1978). *M. bidentata* is one of the few bivalve species that produces dimorphic sperm. Sperm are stored in the suprabranchial chamber in three separate ways: in sac-like spermatophores, within a ventral fold in the floor of the gill chamber (termed the accessory male organ by Deroux), or attached in irregular masses to the abfrontal surfaces of the gill filaments. Ockelmann and Muus (1978) interpreted the accessory male organ as a seminal receptacle and argued that the production of spermatophores and dimorphic sperm indicated that outcrossing normally occurs in this species. The actual mechanism of sperm transfer is still unclear.

Mysella tumida (Carpenter, 1864) occurs in the North Eastern Pacific (Abbott, 1974) and relatively little is known about its reproductive cycle. It broods embryos in the gill chamber, is hermaphroditic, and its monomorphic sperm are atypical in that while in the testis they possess numerous microvilli that radiate from the middle piece (Ó Foighil, 1985b). This study aims to describe how sperm storage is achieved in *M. tumida* and to outline how sperm transfer may occur. The results presented may help explain how outcrossing and sperm storage are achieved by other ctenidial brooding bivalves.

MATERIALS AND METHODS

Specimens of *Mysella tumida* were sampled intertidally at monthly intervals from August 1982 to September 1983 at Patricia Bay, Victoria, British Columbia, Canada. Thirty live individuals (>2.0 mm in valve length) per sample were dissected and examined with a dissecting microscope to determine the reproductive cycle. The incidence of developing embryos in the suprabranchial chamber and of sperm and eggs in the gonad were recorded. An additional thirty specimens were examined each month using light histology to investigate the incidence of sperm attachment to the gill filaments. Specimens were fixed in 2% glutaraldehyde (biological grade), decalcified in a 1:1 mixture of 2% ascorbic acid and 0.3 *M* NaCl (Dietrich and Fontaine, 1975) for 2–4 days, processed by routine methodology, and embedded in paraffin wax. Serial sections at 7 μm intervals were cut and then stained with Eriochrome cyanin (Chapman, 1977).

Gills and spermatophores were dissected from live specimens and fixed for 1 hour at 4°C in a 3:1 mixture of 4% glutaraldehyde and 1% osmium tetroxide in 3% NaCl

(Smith, 1983). They were then dehydrated in an acetone series, critical point dried, gold coated, and viewed with a JEOL JSM-35 scanning electron microscope. Gill filaments and spermatophores were fixed for transmission electron microscopy in 5% glutaraldehyde with 0.1 *M* sodium cacodylate buffer at pH 7.4, and 0.25 *M* sucrose, for one hour at room temperature. They were then rinsed in buffer solution and postfixed in 1% osmium tetroxide in the same buffer for one hour at 4°C. Specimens were dehydrated in an ethanol series, embedded in Epon-812, and sectioned with glass knives on a Reichert ultramicrotome. Silver-grey sections were stained with uranyl acetate and lead citrate, and viewed with a Philips EM-300 transmission electron microscope.

The fertilization efficiencies of 50 individuals of *Mysella tumida* were investigated by dissecting out and examining broods of early embryos (4 cell–blastula stage). Embryonic development within each brood was synchronous and the proportion of unfertilized eggs (characterized by the absence of cleavage or polar bodies) was recorded.

RESULTS

Reproductive cycle

The reproductive cycle of the Patricia Bay *Mysella tumida* population is outlined in Figure 1. Brooding individuals were detected between February and May 1983. There was a corresponding drop in the frequency of animals producing sperm during March and April, and in the number of individuals undergoing oogenesis from March to June. This pattern of seasonal reproduction is set against an environmental background which shows a marked seasonality in ambient temperature. From January to May 1983 some specimens were found to possess large masses of sperm cells attached to their gill filaments. The monthly flux in the frequency of animals with sperm on their gills broadly paralleled, but preceded by one month, the proportion of brooding individuals in the population.

Sperm-gill attachment

The *Mysella tumida* gill is composed of a pair of inner demibranchs, each formed by a descending and ascending lamella (Fig. 2). Both lamellae possess frontal and lateral cilia, and latero-frontal cirri, but differ in that the abfrontal surface of the ascending lamella is not ciliated (Fig. 3), whereas the descending lamella bears abfrontal cilia (Fig. 4). The ascending lamella is also the larger of the two. Sperm attachment occurs at the abfrontal surface of the constituent gill filaments in the ascending lamella. The attached sperm are typically aggregated to form distinct patches on the gill surface (Fig. 5). Within these patches, the gill filaments and ostia are obscured by the sperm flagella (Fig. 6) and the sperm heads are orientated with the acrosomal end facing the gill filaments (Fig. 7). The attached sperm cells are limited in their distribution to the abfrontal surface of the gill filament by the lateral cilia (Fig. 8).

Despite being orientated toward the gill filaments, the sperm heads are usually separated from the gill epithelium by a 2–8 μm gap. The gap between the sperm heads and the gill epithelium is spanned by fine thread-like processes (Fig. 9), which are extensions of the sperm plasmalemma where it lies closely apposed to the intact acrosomal vesicle (Fig. 10). These extensions are 30 nm in diameter and resemble the microvilli which radiate from the middle piece of *Mysella tumida* sperm while in the testis (Ó Foighil, 1985b). However, no microvilli are present on the middle piece of

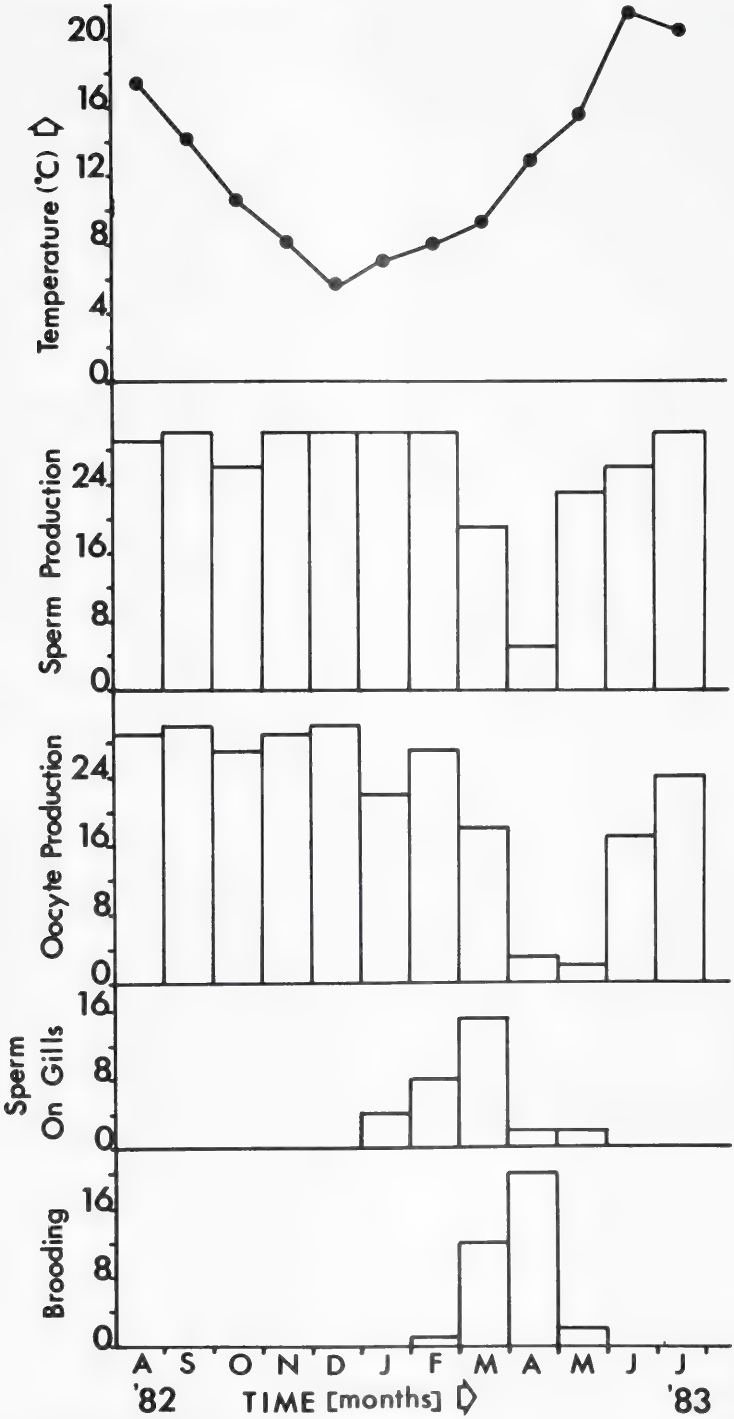
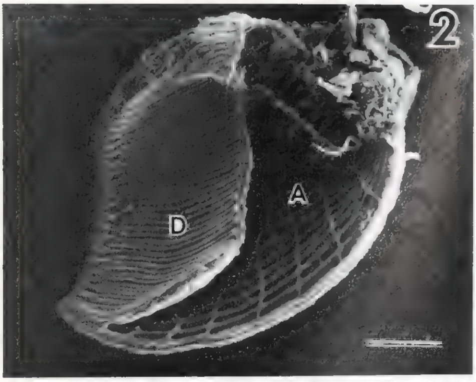
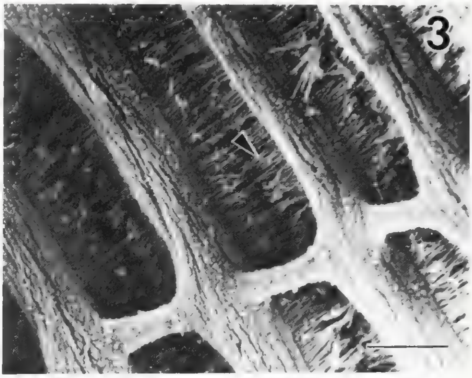
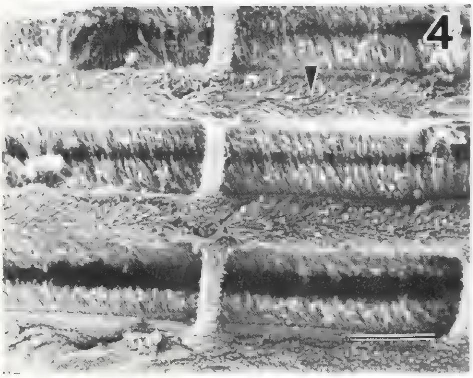
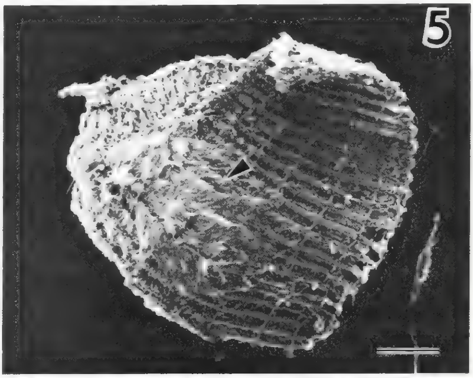


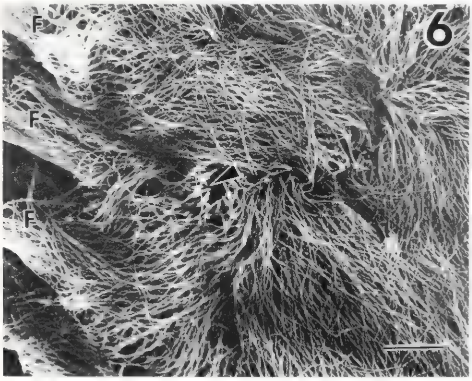
FIGURE 1. Outline of reproductive cycle of *Mysella tumida* at Patricia Bay from August 1982 to July 1983. Ambient surface water temperatures and the reproductive condition of 30 individuals per monthly sample is presented.











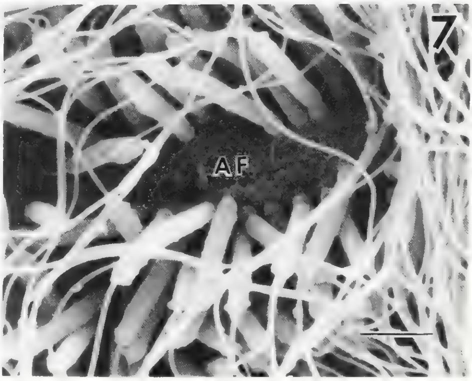


FIGURE 2. Scanning electron micrograph (S.E.M.) of *M. tumida* gill demibranch. A, ascending lamella; D, descending lamella. Scale = 100 μm.

FIGURE 3. S.E.M. of abfrontal surface of *M. tumida* ascending gill lamella. Note absence of abfrontal cilia. Arrow points to lateral cilia. Scale = 10 μm.

FIGURE 4. S.E.M. of abfrontal surface of *M. tumida* descending gill lamella. Arrow points to abfrontal cilia. Scale = 10 μm.

FIGURE 5. S.E.M. of *M. tumida* ascending gill lamella with attached sperm mass (see arrow). Scale = 100 μm.

FIGURE 6. S.E.M. of *M. tumida* sperm mass attached to gill filaments showing occlusion of ostia by sperm flagella. Arrow points to sperm heads. F, gill filaments. Scale = 30 μm.

FIGURE 7. S.E.M. of attached *M. tumida* sperm heads. Note orientation of acrosomal end toward abfrontal gill epithelium (AF). Scale = 5 μm.

M. tumida gill-attached sperm (Fig. 11). On reaching the abfrontal surface of the gill filaments, the acrosomal microvilli interdigitate with the gill filament microvilli (Fig. 12). The gill epithelium microvilli are shorter in length (<1.25 μm) and greater in diameter (up to 125 nm) than the acrosomal microvilli, so that both types may be readily distinguished. Membrane fusion between the two microvillar types was not observed, but they frequently come into close apposition (<14 nm) where their respective glycocalices make contact (Fig. 13).

Living gills bearing attached sperm were examined by light microscopy. The sperm masses were continually buffeted by powerful water currents generated by the lateral cilia and passed in through the gill ostia. It was not possible to determine if the constant flexing of these sperm cells was caused solely by the action of the lateral cilia. However, smaller masses of sperm attached to more sheltered portions of the gill chamber demonstrated a low level of flagellar movement by individual sperm cells. This indicates that, although tethered by microvilli, the attached sperm remain activated.

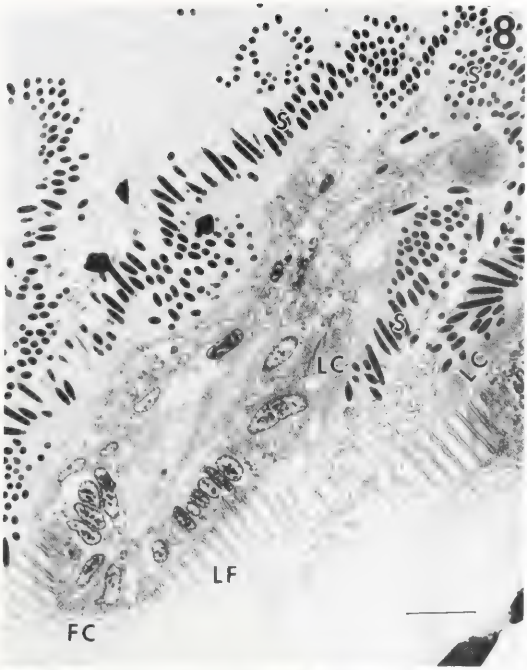
Eggs were fertilized by the stored spermatozoa after being spawned into the gill chamber. Brooding individuals were not observed to have sperm attached to their gill filaments. The fecundity of 50 individuals ranged from 221–1279 (number of eggs spawned) per individual. A total of 40 unfertilized eggs were detected among 39,660 developing embryos. This is equivalent to a mean fertilization efficiency of 99.9%.

Spermatophore production and transfer

Histological examination of animals collected to determine the reproductive cycle revealed spermatophores in the suprabranchial chambers of two individuals in February 1983. In both cases the spermatophores projected from the gonaduct opening into the gill chamber (Fig. 14). From January to March 1984 freshly collected *Mysella tumida* were held in finger bowls of seawater at 10°C and checked daily for evidence of sperm transfer. Fourteen individuals were observed releasing single spermatophores *via* their posterior exhalent siphons. They were elongate, delicate, transparent structures (Fig. 15), up to 0.6 mm in length, which floated freely in the finger bowls. Spermatophores contained masses of sperm cells (Fig. 16), many of which demonstrated a low level of flagellar activity, some spermatids and occasional oocyte fragments enclosed within a 0.2–0.6 μm thick wall. The spermatophore wall had no distinct substructure, and was composed of a layer of flocculant material with frequent electron translucent pockets (Fig. 17). No opening was discerned in the spermatophore wall. Some spermatophoric sperm had microvilli radiating from their acrosomal ends as well as from the middle piece (Fig. 18).

Newly released spermatophores were placed together with single specimens of *Mysella tumida* in finger bowls to determine if spermatophore uptake would occur. On contacting the extended foot of these highly mobile bivalves, the spermatophores were passed posteriorly by the cilia of the foot surface. Upon foot withdrawal, the spermatophores adhered to the shell surface, typically in the posterior-ventral region of the valves (Fig. 19). This was frequently (>20 occasions) observed in freshly collected individuals. In this position the spermatophores occlude the exhalent opening which leads into the suprabranchial chamber. Laboratory-held specimens retained the externally attached spermatophore for up to 4 days. During this time the innermost portion of the spermatophore wall that projected between the open valve margins was ruptured by occasional adductor muscle contractions. Sperm cells were observed being sucked into the suprabranchial chamber following sudden adductor muscle relaxations.

Spermatophores were not taken into the mantle cavity through the anterior in-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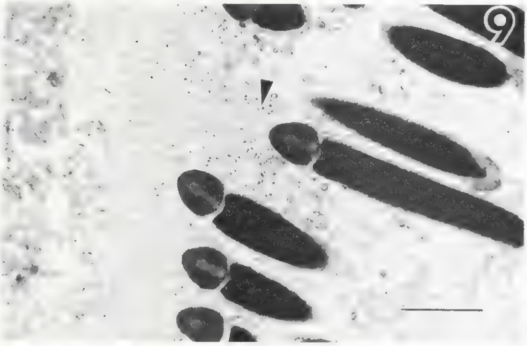
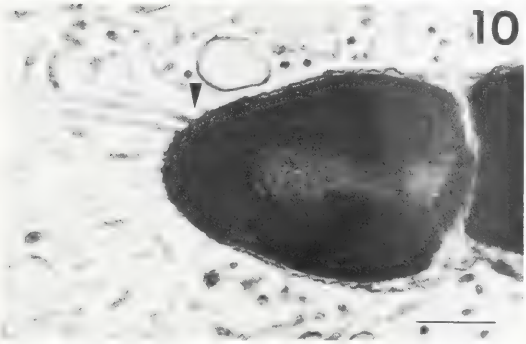
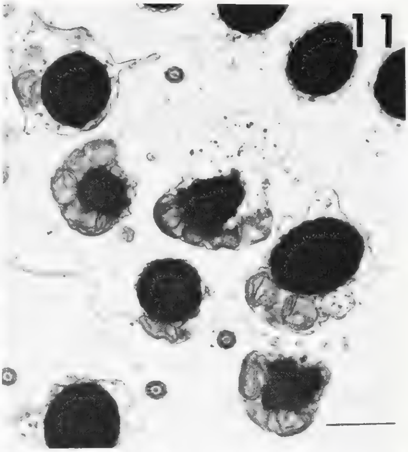
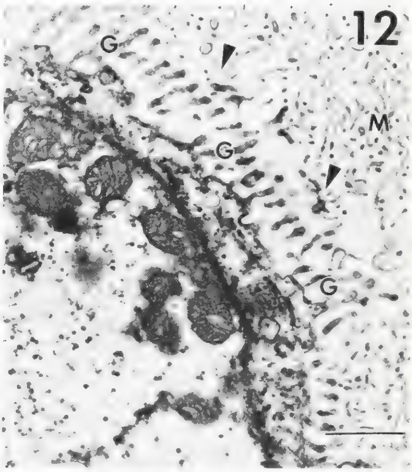
FC









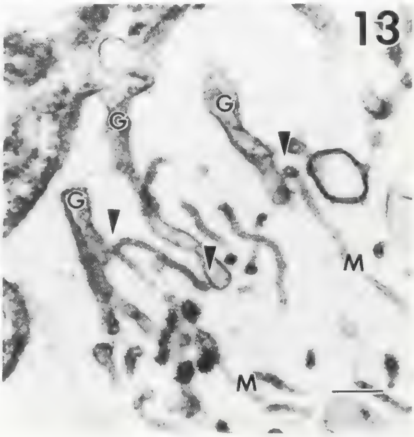


FIGURE 8. Transmission electron micrograph (T.E.M.) of cross-section through *M. tumida* gill filament with attached spermatozoa. FC, frontal cilia; LC, lateral cilia; LF, laterofrontal cirri; S, attached spermatozoa. Scale = 10 μm.

halent/pedal opening and sperm masses placed in the inhalent current did not pass to the suprabranchial chamber, but were transported to the mouth and ingested as described by Ockelmann and Muus (1978) for *Mysella bidentata*.

DISCUSSION

Sperm storage and brooding are closely coordinated seasonal events in the reproductive cycle of *Mysella tumida*. The advent of sperm storage in the Patricia Bay population precedes by one month the onset of brooding. Brooding is also a seasonal phenomenon in *M. bidentata* populations (Ockelmann and Muus, 1978; Ó Foighil *et al.*, 1984), but individuals storing sperm are prevalent throughout most of the year (Ockelmann and Muus, 1978). The difference between the two species in the duration of sperm storage may reflect the more specialized sperm storage microenvironment found in *M. bidentata*. This involves a possible nutritive role for the oligopyrene sperm and the development of a distinct seminal receptacle (Ockelmann and Muus, 1978). *M. bidentata* sperm may also attach to the abfrontal surface of gill filaments, but the presence of two sites for sperm attachment in this species is of unknown significance.

While in the testis, *Mysella tumida* sperm microvilli are located on the middle piece (Ó Foighil, 1985b). After being packaged into spermatophores and released through the exhalent opening some sperm cells develop microvilli at the acrosomal end. Sperm cells dissected live from the gonad also develop acrosomally placed microvilli (pers. obs.). The relocation of the sperm microvilli may occur with sperm activation. It may be significant that in the two positions where the microvilli occur, the plasmalemma comes into close apposition (<14 nm) to the membranes of the underlying organelles, the acrosomal vesicle and mitochondria. Acrosomally placed microvilli offer some potential advantages over those located on the middle piece in achieving sperm attachment to the gill epithelium. In the former case, attachment cannot be disrupted by the undulating flagellum and a greater number of sperm cells may adhere per area of gill epithelium because the sperm are orientated with their long axis perpendicular and not parallel to the gill surface. The rod-like shape of the sperm head in *M. tumida* (Ó Foighil, 1985b) may also allow a more efficient packing of spermatozoa in spermatophores and on gill filament epithelia. *M. bidentata* eupyrene sperm heads are also rod-like in shape (Ockelmann and Muus, 1978).

Mysella tumida sperm are constantly buffeted by the water currents generated by the nearby lateral cilia, but remain firmly attached to the ascending gill filaments until the eggs are spawned into the suprabranchial chamber. Sperm-gill adhesion is perhaps achieved by glycoprotein crosslinking of the epithelial cell and sperm cell glycocalices. Similar forms of sperm storage have been discovered in a variety of invertebrate and

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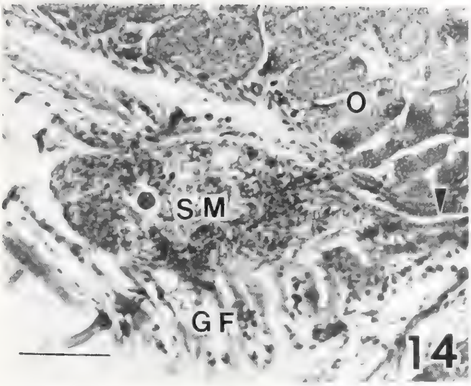
FIGURE 9. T.E.M. of longitudinal section through *M. tumida* sperm heads, revealing numerous microvilli (arrow) radiating from acrosomal region of spermatozoa toward gill epithelium. Scale = 2 μm.

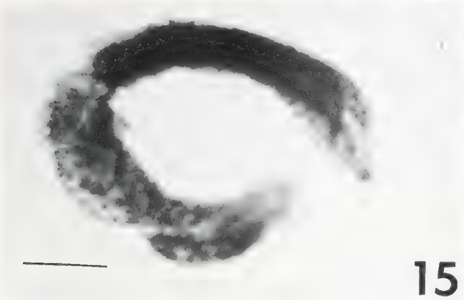
FIGURE 10. T.E.M. of median longitudinal section through *M. tumida* acrosomal vesicle. Sperm cell microvilli are apparent as extensions of the plasmalemma (arrow) where it comes into proximity with underlying acrosomal vesicle. Scale = 0.4 μm.

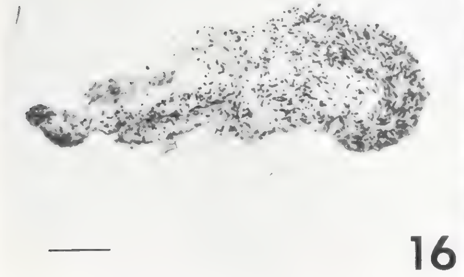
FIGURE 11. T.E.M. of section through middle pieces of attached *M. tumida* spermatozoa. Note absence of microvilli. Scale = 1 μm.

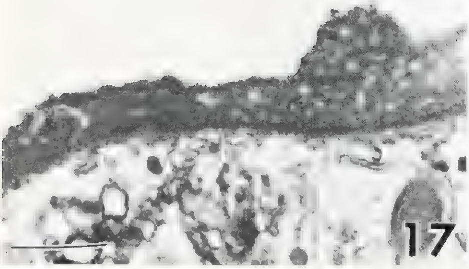
FIGURE 12. T.E.M. of section through *M. tumida* gill filament epithelial cell surface showing interdigitation (arrows) of gill filament microvilli (G) and spermatozoan microvilli (M). Scale = 1 μm.

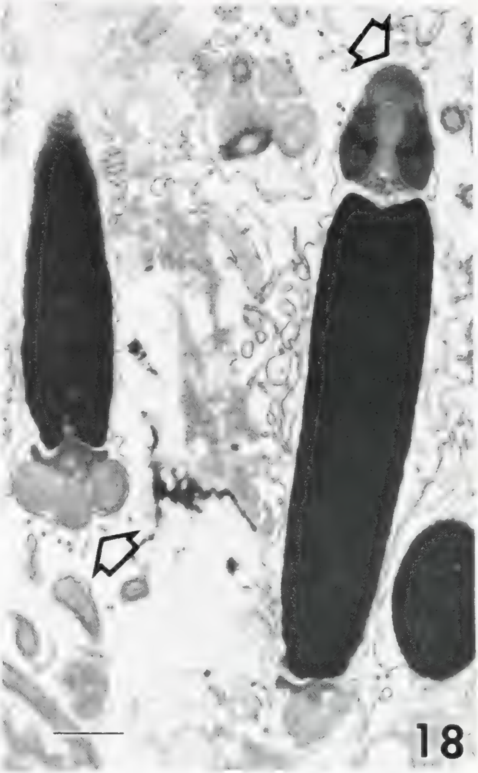
FIGURE 13. T.E.M. of section through *M. tumida* gill filament epithelial cell surface revealing the close apposition of gill filament microvilli (G) and spermatozoan microvilli (M). Arrows indicate areas of close contact between both microvillar types. Scale = 0.2 μm.











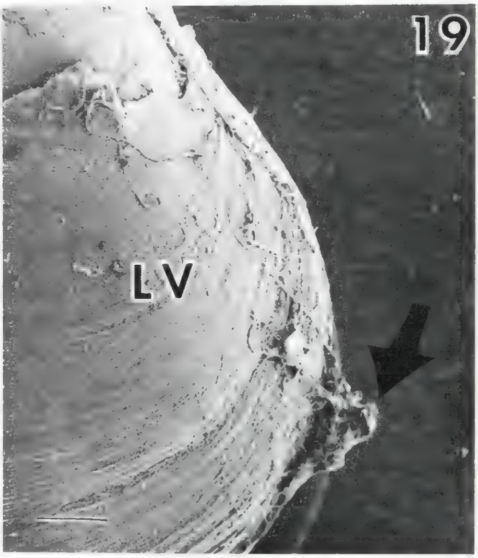


FIGURE 14. Light micrograph (L.M.) of longitudinal section through *M. tumida* spermatophore, projecting from the gonoduct (arrow) into suprabranchial chamber. GF, gill filaments; O, developing oocytes in gonad; SM, spermatophore. Scale = 100 μm.

FIGURE 15. L.M. of newly released *M. tumida* spermatophore. Scale = 80 μm.

FIGURE 16. L.M. of cross-section through *M. tumida* spermatophore. Note numerous spermatozoa enclosed by thin spermatophore wall. Scale = 40 μm.

vertebrate species *e.g.*, the polychaete *Spirorbis spirorbis* (Daly and Golding, 1977; Picard, 1980), the gastropod *Cochlostoma montanum* (Giusti and Selmi, 1985), the reptile *Thamnophis sirtalis* (Hoffman and Wimsatt, 1972), and a variety of bat species (Racey and Potts, 1970; Racey, 1979; Mori *et al.*, 1982; Andrucetti *et al.*, 1984).

Spermatophore production in the Bivalvia is known to occur in a small number of galeommatacean species (Ó Foighil, 1985a), which includes *Mysella bidentata* (Deroux, 1961; Ockelmann and Muus, 1978). Deroux (1961) concluded that *M. bidentata* spermatophores are formed by the specialized sperm-storing portion of the gill that he termed the accessory male organ. Ockelmann and Muus (1978) suggest that due to the elongate shape of the spermatophores and their typical position opposite the genital pore in the suprabranchial chamber, that in this species the spermatophores are formed in the gonadal duct. This interpretation is supported by observations on *M. tumida* where there is no accessory male organ and spermatophores have been observed protruding from the gonadal ducts. It is probable that the spermatophore wall is secreted by the gonadal duct epithelium and is moulded around the emerging sperm mass. In the polychaete species *Polydora ligni* and *P. websteri*, spermatophores are formed in the nephridia where sperm masses are surrounded by a layer of adherent microvilli shed from the nephridial epithelium (Rice, 1980). The spermatophore wall of *M. tumida* differs in that it is composed of amorphous material, which would indicate an alternate mode of construction.

Spermatophore production in the Galeommatacea occurs in species which are normally aggregated, usually on or around a host animal (Ó Foighil, 1985a). *Mysella tumida* has been previously found in the oxygenated sediment zone surrounding burrows of the holothurian *Leptosynapta clarki* (Ó Foighil and Gibson, 1984). In Patricia Bay *M. tumida* are typically clustered around the ends of the tubes of the polychaete *Mesochaetopterus taylori.* Up to 120 individuals may occupy a small volume of sediment around the tube of an individual *Mesochaetopterus* (pers. obs.).

The aggregated nature of the *M. tumida* populations provides feasible conditions for sperm transfer to occur *via* spermatophores. Laboratory observations on newly sampled specimens indicate that spermatophore release through the exhalent opening occurred in the Patricia Bay population from January to April in 1984. Although not all the details of sperm transfer in this species are yet obvious, it is clear that once released from the suprabranchial chamber, sperm can re-enter the gill chamber only through the exhalent opening. *M. tumida* is an indirect deposit feeder (pers. obs.) and uses its foot during the feeding process in a manner similar to *M. bidentata* (Ockelmann and Muus, 1978). Spermatophores contacting the foot become attached to the posterior-ventral shell margin and occlude the exhalent opening. Adductor muscle contractions lead to the rupturing of the spermatophore wall and the inhalation of sperm, but subsequent events involving the attachment of the spermatozoa to the gill filaments were not observed. For this to occur, some behavioral modification of the normal suprabranchial water flow, most likely a reduction in the activity of the lateral cilia, is necessary.

Spermatophores, temporary dwarf males and complemental males are utilized as methods of bulk sperm transfer in the Galeommatacea (Ó Foighil, 1985a). The specialized mode of sperm transfer in *Mysella bidentata* involves the formation of di-

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FIGURE 17. T.E.M. of section through spermatophore wall. Scale = 0.4 μm.

FIGURE 18. T.E.M. of longitudinal section through *M. tumida* spermatophoric sperm cells. Microvilli are present at both the acrosomal end and the middle piece (arrows). Scale = 0.9 μm.

FIGURE 19. S.E.M. of posterior-ventral region of *M. tumida* valves. Note spermatophore (arrow) lodged between valve margins. LV, left valve. Scale = 100 μm.

morphic sperm, spermatophores, and seminal receptacles and may result in enhanced fertilization success (Ockelmann and Muus, 1978). *M. tumida* achieves a high degree of fertilization efficiency (99.9%) by employing some of these specializations; *e.g.* bulk sperm transfer of spermatozoa followed by sperm storage at the fertilization site until the eggs are spawned. It seems likely that other Galeommatacean species that undergo bulk sperm transfer benefit from a similarly high fertilization success. Recent *in situ* work on spawning in echinoids indicates that in broadcast spawners, zygote production could be much less than egg production, unless the aggregation of synchronous spawners counter-acts excessive sperm dilution (Pennington, 1984). *M. tumida* produces a small amount of sperm relative to broadcast spawners, but may avoid the reproductive hazards of excessive sperm dilution by fertilizing the eggs in a correspondingly small water body—the suprabranchial chamber.

Localized fertilization in or on the parent animal is an obvious prerequisite for the brooding habit and bulk sperm transfer is an effective method of achieving localized fertilization. Therefore, the development of bulk sperm transfer methods may have preceded the brooding habit in many outcrossing invertebrates. In bivalves these methods include pseudocopulation (Townsley *et al.,* 1965) and sperm ball production (Coe, 1931; Andrews, 1979) as well as the aforementioned reproductive specializations. A possible advantage for localized fertilization is that it results in a greater degree of fertilization efficiency, which is proportionally more important for animals of low fecundity. An alternate method of achieving localized fertilization is through selfing, which has been reported from a variety of bivalve species (Thomas, 1959; Castanga and Duggan, 1971; Chanley and Chanley, 1980; Morton, 1980; Kraemer, 1983). There is indirect evidence that *Mysella tumida* normally outcrosses: (1) the almost equal male and female investment in the gonad (Ó Foighil, 1985b) which is indicative of outcrossing (Heath, 1979); (2) the production and release of spermatophores; and (3) the occurrence of sperm storage even though this species is a simultaneous hermaphrodite. The possibility of facultative selfing in *M. tumida* as reported from *Corbicula fluminea* (Kraemer, 1983), however, is not ruled out.

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SUSPENSION OF MARINE NEMATODES IN A TURBULENT TIDAL CREEK: SPECIES PATTERNS*

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ABSTRACT

Nematodes had a mean abundance of hundreds per m^3 in an estuarine creek. They were four orders of magnitude less abundant in the water column than in the sediment. The water column nematode assemblage was dominated by three species of *Metachromadora* which comprised 57% of the suspended nematodes. Other abundant species were *Chromadorita* aff. *minima* and *Ptycholaimellus pandispiculatus.* Numbers of suspended nematodes were highest during the ebb and flood tides when currents were fastest. The two factors that appear to be most important in determining which species are in the water are the vertical distribution and the overall abundance of a species in the sediment. Surficial distributions and high sediment abundance will result in greater representation in the water column. Suspension in the water column and subsequent transport by tidal currents potentially plays a significant role in the local dispersal of certain meiobenthic nematode species. Corollaries to water column dispersal are an improved ability of nematodes in colonizing new habitats, an increased diversity in some habitats as animals are carried between habitats, and an interaction between hydrodynamic factors and benthic topography that affects small-scale spatial distribution.

INTRODUCTION

Until recently, the occurrence of marine free-living nematodes in the water column had not been systematically investigated and data on suspended nematodes were sparse, serendipitous, and anecdotal (Gerlach, 1977). Several recent quantitative studies have demonstrated the consistent occurrence of nematodes in the water column. Nematodes are numerous in sediments but occur in comparatively low abundance in the water column (*e.g.*, Bell and Sherman, 1980; Sibert, 1981; Jacobs, 1984 and references therein; Palmer and Gust, 1985); however, the consistent occurrence of even relatively low numbers in the water column has significant implications for our concepts of nematode dispersal, colonization, diversity, and spatial patchiness.

Palmer and Gust (1985) showed that the water column dispersal of nematodes from tidal mudflats was primarily the result of their passive erosion and suspension. For a few species, active swimming may play a role (Gerlach, 1977; Jensen, 1981) but as hypothesized by Warwick and Gee (1984) for *Ptycholaimellus ponticus,* nematodes with a shallow distribution in the sediment may be transported in the water following erosion. In the Palmer and Gust study, nematodes were the numerically dominant meiofaunal taxon in the sediment (~62%) and in the water (~30%). Over a complete tidal cycle 5% of the total sediment nematodes may be suspended; however, at any

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one instant in time less than 1% are in suspension (Palmer and Gust, 1985). The percent of sediment fauna suspended was significantly lower for nematodes than for the copepods, foraminiferans, or bivalves (Palmer and Gust, 1985). Nematodes typically have the highest sediment abundance so even a small percentage suspended, results in a large number of nematodes in the water. Palmer and Gust suggested that the low percentage suspended was the result of nematodes behaviorally avoiding the sediment surface and thereby reducing their susceptibility to suspension. In a study of meiofauna behavior, Palmer (1984) found that nematodes rarely emerged from the sediment. When nematodes were artifically suspended (*sensu* Palmer, 1984), 64% were transported downstream at low current velocities (6–8 cm/s) thus, once in suspension, only 36% of the suspended nematodes were able to re-enter the sediment or maintain their position on the sediment surface.

Studies of the vertical distribution of nematodes in marine sediments have demonstrated predictable patterns for certain taxa. Some genera will typically have representatives deep in the sediment while others are only found near the surface (*e.g.*, Fenchel *et al.*, 1967; Boaden and Platt, 1971; Joint *et al.*, 1982; and references in Table I). A simple model of passive suspension predicts that those nematodes abundant in the upper layers of sediment should be the most abundant ones in the water column in proportion to their sediment abundance. Behavioral adaptations however, may certainly reduce passive suspension. For copepods, flow-related behaviors vary among species (Palmer, 1984) and transport in the water column also varies among species (Hagerman and Rieger, 1981; Palmer and Gust, 1985). In the latter study, there was a much higher copepod species diversity in the water than in the sediment with at least 50% of the suspended copepods belonging to species whose centers of distribution were considerable distances away from the study site. Thus, over half of the total copepods in the water were advected from other marsh areas and not locally eroded. Such findings are contingent on careful identification of fauna to species level. Here we report on nematode species patterns from the study of Palmer and Gust (1985).

TABLE I

Relative vertical distribution of species or congeners listed in this study.

Depth in sediment (cm) Species	0–1	1–2	>2	Reference
	% at depth			
Metachromadora obesa[1]	95	3	2	Ott, 1972
M. vivipara	80–100	—	—	Platt, 1977
M. vivipara[2]	~90	10	—	Warwick and Gee, 1984
M. sueica	93	5	2	Platt, 1977
Ptycholaimellus pandispiculatus[1]	70	20	10	Ott, 1972
P. ponticus[2]	93	7	—	Warwick and Gee, 1984
Anoplostoma viviparum	80–100	—	—	Platt, 1977
Terschellingia longicaudata[3]	40	40	20	Ott, 1972
T. longicaudata[2]	41	49	—	Warwick and Gee, 1984
Sabatieria pulchra[2]	32	68	—	Warwick and Gee, 1984
S. cupida	20–80	—	—	Platt, 1977
Odontophora setosa	20–80	—	—	Platt, 1977

[1] Estimated from Figure 6.
[2] Estimated from Figure 8.
[3] Estimated from Figure 7.

Specifically, our goals were to answer the following questions:

(1) Does the percentage of a nematode species in suspension vary significantly from its proportional representation in the sediment? If so, can this be related to our knowledge of species-specific behaviors or vertical distribution patterns?

(2) Does the species composition of the suspended nematodes differ from the composition of the mudflat fauna? If so, what fraction of the suspended nematodes are advected?

(3) Are there differences in the suspension of juveniles *versus* adults or males *versus* females?

MATERIALS AND METHODS

The sample site was an unvegetated mudflat in the North Inlet Estuary, near Georgetown, South Carolina (33°20'N, 79°10'W). Samples were collected during a neap tide in June, 1981, and during the following spring tide. Meiofaunal abundances in the sediment and in the water column were measured simultaneously at 23 times over two, 48-hour periods. Details of the sampling scheme are described in Palmer and Gust (1985). Briefly, replicate water samples were collected at 17, 37, and 57 cm above the bottom using slow-velocity pumps. The number of animals reported here is the product of the proportion of each species in the aliquot of identified individuals and the total number of nematodes counted in that sample. All faunal abundances are corrected for changes in volume of water over the mudflat as described in Palmer and Gust (1985). The final figures reported are the number of animals per cubic meter of water. Samples for spring and neap tides were combined (104 water samples, 23 sediment samples) since Palmer and Gust (1985) found no spring/neap differences for total nematodes. Calculations of total nematode abundance in the water are considered conservative since sediment-water interface data were not included (Palmer and Gust, 1985). Concurrently with pump samples, sediment samples were taken with a 2.5 cm diameter corer to a depth of 1.5 cm (7.36 cm^3 of sediment) and adjusted to number per m^3. This depth is well below the redox potential discontinuity zone (which was <1 cm in June) and would include most of the nematodes present (Coull and Bell, 1979).

Nematodes were fixed at the time of collection in borax buffered formalin and stained with Rose Bengal. Every second animal was removed from the sample, dehydrated, and mounted in anhydrous glycerine (Seinhorst, 1959) for identification to species and sex or life stage (male, female, juvenile). Palmer and Gust (1985) give a full presentation of the results for all major taxa and the hydrodynamic results of interest in testing the hypothesis of active water-column entry *versus* passive erosion.

RESULTS

Nematodes were approximately four orders of magnitude less abundant in the water column than in the sediment. The three most abundant nematodes in the water column were all species of the genus *Metachromadora* (Desmodoridae). *Metachromadora (Metachromadora) chandleri* (Chitwood, 1951) and *Metachromadora (Neonyx) obesa* Chitwood, 1936 were co-dominant in the water column, but only ranked thirty-third and thirty-fifth in abundance among the sediment fauna (Table II). *Metachromadora (Metachromadoroides) remanei* Gerlach, 1951, was third in abundance in the water but ranked seventh in the sediment. Five and one half percent of the sediment abundance of *M. chandleri* and *M. obesa* were in suspension. Although *M. remanei,*

TABLE II

Composition and abundance of suspended and sediment nematode assemblages

	Water column			Sediment				
Taxon	Mean	SE	Dom	Mean	SE	Dom	Rank	%
Metachromadora chandleri	136	(17)	24.3	2303	(2303)	0.2	35	5.9
Metachromadora obesa	122	(16)	24.6	2366	(2367)	0.2	33	5.2
Metachromadora remanei	42	(8)	8.1	31215	(7706)	4.1	7	0.1
Chromadorita aff. *minima*	42	(9)	9.6	373	(373)	0.2	45	11.3
Ptycholaimellus pandispiculatus	29	(5)	5.7	339600	(46877)	34.3	1	<0.1
Viscosia papillata	8	(3)	1.3	51281	(11417)	6.8	6	<0.1
Daptonema erectum	7	(3)	1.9	2138	(1479)	0.9	36	0.3
Daptonema sp. 1	7	(2)	1.6	0	(0)	0	—	—
Daptonema spp.	6	(4)	0.7	11564	(4474)	1.2	12	0.1
Desmodora cepalata	6	(2)	1.2	3616	(2251)	0.5	25	0.2
Microlaimus spp.	5	(2)	1.5	993	(993)	0.1	43	0.5
Chromadoridae spp.	5	(2)	1.1	1019	(1019)	0.1	40	0.5
Spilophorella paradoxa	4	(2)	1.5	928	(928)	0.1	44	0.5
Terschellingia longicaudata	4	(2)	0.8	62728	(18757)	6.7	5	0.4
Sphaerolaimus sp. 1	3	(1)	1.0	8760	(3554)	1.1	15	<0.1
Oncholaimidae sp. 1	3	(1)	0.7	0	(0)	0		—
Anoplostoma cf. *viviparum*	3	(2)	0.4	13573	(6844)	1.0	9	<0.1
Linhomoeidae spp.	3	(1)	0.5	1019	(1019)	0.1	41	0.3
Sabatieria spp.	3	(1)	1.9	7706	(4647)	0.7	17	<0.1
Leptolaimidae sp. 1	2	(1)	0.5	4856	(3453)	0.3	20	<0.1
Desmodoridae spp.	2	(1)	0.3	0	(0)	0	—	—
Oncholaimoides striatus	2	(2)	0.6	0	(0)	0	—	—
Theristus spp.	2	(1)	0.5	7290	(6011)	0.7	18	<0.1
Graphonema sp.	2	(1)	0.6	0	(0)	0	—	—
Sabatieria pulchra	2	(1)	0.4	97533	(19218)	11.9	2	0.1
Metachromadora spp.	2	(1)	0.6	0	(0)	0	—	—
Spirinia spp.	2	(1)	1.0	1098	(1098)	0.1	38	0.2
Dorylaimopsis metatypica	2	(1)	0.3	3764	(2605)	0.3	24	0.1
Eurystomina sp.	2	(1)	0.3	0	(0)	0	—	—
Antomicron sp.	1	(1)	0.2	0	(0)	0	—	—
Halalaimus spp.	1	(1)	0.2	12410	(5696)	0.8	10	<0.1
Cyatholaimidae spp.	1	(1)	0.7	3837	(2904)	0.3	23	<0.1
Leptolaimidae spp.	1	(1)	0.3	2854	(2070)	0.2	28	<0.1
Viscosia brachylaimoides	1	(1)	0.2	1776	(1776)	0.1	37	<0.1
Daptonema sp. 2	1	(1)	0.2	75577	(13133)	8.6	4	<0.1
Others	1	(1)	0.4	1076	(1076)	0.2	39	0.1
Monhysteridae spp.	1	(1)	0.1	2839	(2063)	0.3	29	<0.1
Sigmophoranema sp.	1	(1)	0.8	0	(0)	0	—	—
Marylynnia sp.	1	(1)	0.1	0	(0)	0	—	—
Axonolaimus spinosus	1	(1)	0.3	0	(0)	0	—	—
Odontophora sp. 1	1	(1)	0.3	0	(0)	0	—	—
Tripyloides sp.	1	(1)	0.4	10192	(3215)	1.3	13	<0.1
Comesomatidae spp.	1	(1)	0.2	0	(0)	0	—	—
Sabatieria americana	1	(1)	<0.1	373	(373)	0.2	46	0.3
Theristus sp. 1	1	(<1)	0.3	0	(0)	0	—	—
Odontophora sp. 2	1	(<1)	0.3	81246	(20178)	7.7	3	<0.1
Parasphaerolaimus sp.	1	(1)	0.1	8488	(3634)	0.8	16	<0.1
Adoncholaimus cf. *thalassophygas*	3	(1)	1	22735	(8065)	2.6	8	0.1
Enoplolaimus sp. 1	1	(1)	0.1	0	(0)	0	—	—
Terschellingia sp. 2	1	(1)	<0.1	2874	(2045)	0.2	27	<0.1
Metachromadora setosus	<1	(<1)	0.2	0	(0)	0	—	—

TABLE II (*Continued*)

Taxon	Water column Mean	SE	Dom	Sediment Mean	SE	Dom	Rank	%
Oxystomina spp.	<1	(<1)	0.5	4815	(4815)	0.3	21	<0.1
Dracograllus sp.	<1	(<1)	<0.1	0	(0)	0	—	—
Oncholaimidae spp.	<1	(<1)	0.1	0	(0)	0	—	—
Xyala sp.	<1	(<1)	0.1	0	(0)	0	—	—
Chromadoridae sp. 1	<1	(<1)	<0.1	2366	(2367)	0.2	32	<0.1
Oncholaimus sp.	<1	(<1)	0.1	0	(0)	0	—	—
Nygmatonchus sp. 1	<1	(<1)	0.1	0	(0)	0	—	—
Enoplolaimidae spp.	<1	(<1)	0.1	0	(0)	0	—	—
Desmodoridae sp. 1	<1	(<1)	0.1	0	(0)	0	—	—
Cyatholaimidae sp. 2	<1	(<1)	<0.1	0	(0)	0	—	—
Rhynchonema sp.	<1	(<1)	<0.1	0	(0)	0	—	—
Cobbia sp.	<1	(<1)	0.1	0	(0)	0	—	—
Calyptronema cf. *maxweberi*	0	(0)	0	3413	(1705)	0.9	26	—
Ironidae sp.	0	(0)	0	3913	(2280)	0.5	22	—
Laimella filipjevi	0	(0)	0	9313	(4249)	1.0	14	—
Laimella sp.	0	(0)	0	993	(993)	0.1	42	—
Sabatieria celtica	0	(0)	0	2718	(2085)	0.4	30	—
Sphaerolaimus sp. 2	0	(0)	0	2367	(2367)	0.1	34	—
Terschellingia sp. 1	0	(0)	0	11961	(9335)	0.9	11	—
Theristus 17s	0	(0)	0	5007	(2933)	0.4	19	—

The mean number of animals, the standard error of the mean (SE), and dominance (mean percent species representation in each sample) are shown. Percent is the percent of sediment nematodes in the water column. For the water column, species are listed in descending order of mean number of animals per m^3. For sediment species, ranks are according to a species' abundance per m^3. Authorities for species are listed in Gerlach and Riemann, 1973, 1974.

was less dominant in the water than the other two *Metachromadora* species, only 0.1% of *M. remanei's* sediment abundance was suspended as compared to ~5% for *M. chandleri* and *M. obesa*.

Juveniles represented the largest proportion of the *Metachromadora* species in the water column (76–92%) (Table III). Local sediment abundance of *M. chandleri* and

TABLE III

Mean sample percentages of females (F), males (M), and juveniles (J) for those species abundant (>1%) in the water column

Species	F	M	J	n
Metachromdora chandleri	9.5	14.0	76.3	(75)
Metachromadora obesa	3.3	4.6	92.1	(79)
Metachromadora remanei	8.3	11.2	80.4	(49)
Chromadorita aff. *minima*	39.2	53.2	7.6	(48)
Ptycholaimellus pandispiculatus	39.4	22.8	37.8	(41)
Viscosia papillata	41.7	16.7	41.7	(12)
Daptonema erectum	72.7	27.3	0	(11)
Daptonema sp. 1	15.4	57.7	26.9	(13)
Desmodora cephalata	33.3	11.1	55.6	(9)
Spilophorella paradoxa	40.0	50.0	10	(10)

n = number of samples in which the species abundance was not equal to zero.

M. obesa were too low to determine sex ratios or adult:juvenile ratios, but for *M. remanei,* 69% of the animals in the sediment were juveniles in comparison to 80% in the water.

A high proportion (11.3%) of the sediment abundance of *Chromadorita* aff. *minima* (Kreis, 1929) was suspended (Table II). In contrast to the *Metachromadora* species, most suspended *C. minima* were adults (39% females, 53% males, and 8% juveniles) (Table III). Most *C.* aff. *minima* were suspended on the ebb tide, and fewest at slack tide (Table IV).

The most abundant sediment dweller, *Ptycholaimellus pandispiculatus* (Hopper, 1961) only made up 6% of the nematodes in suspension (Table II).

Five species [*Viscosia papillata* Chitwood, 1951; *Daptonema erectum* (Wieser and Hopper, 1967); *Daptonema* sp. 1; *Desmodora (Pseudochromadora) cephalata* Cobb, 1920; and *Spilophorella paradoxa* (DeMan, 1888)] each comprised 1–2% of the water column assemblage. *Daptonema erectum, Desmodora cephalata,* and *S. paradoxa* were represented by 0.2–0.5% of their sediment abundance. *Daptonema* sp. 1 apparently was transported from another area because it was not present in the sediments at the experimental site. *Viscosia papillata* had a moderate to high sediment abundance, but was not proportionately common in the water column. The remaining species (Table II) were too rare to discuss in detail.

When currents were fastest *i.e.,* mid-ebb and flood tides (Palmer and Gust, 1985), more nematodes were suspended (Table IV). The *Metachromadora* species in particular had a wide disparity between ebb/flood and slack tides.

DISCUSSION

The *Metachromadora* species are thick bodied with numerous stiff setae and conspicuous lateral alae (wing-like extensions of the cuticle along the length of the body). *M. chandleri* (= *parasitifera* Timm, 1952) is a lethargic species described from rich detrital mud (Timm, 1952) but *M. obesa* is common on sand surfaces (Ott, 1972). Other species of *Metachromadora* for which the vertical distribution has been inves-

TABLE IV

Nematode mean abundance in the water column (per m³) during ebb, flood, and slack currents of neap and spring tides. Data given for dominant species (>1% only)

	Neap			Spring		
Species	Flood	Slack	Ebb	Flood	Slack	Ebb
	n = 24	12	19	20	10	19
Metachromadora chandleri	150	14	194	192	40	131
Metachromadora obesa	130	5	152	170	34	142
Metachromadora remanei	40	4	96	37	14	23
Chromadorita aff. *minima*	33	16	91	30	16	45
Ptycholaimellus pandispiculatus	10	0	32	24	18	36
Viscosia papillata	10	0	15	11	1	3
Daptonema erectum	1	3	20	0	0	18
Daptonema sp. 1	5	3	7	9	0	16
Desmodora cephalata	1	0	10	3	29	3
Spilophorella paradoxa	2	1	6	6	7	2

See Table I for magnitude of variation (SE).
n = number of samples processed.

tigated are also found near or at the surface (Platt, 1977; Blome, 1983). Bell and Sherman (1980) found *M. pulvinata* to be third most abundant in their water column samples taken near (0.5 km) our study site and Sherman and Coull (1980) found that *M. pulvinata* and *M. remanei* were capable of recolonizing a disturbed site (0.5 km from our site) within one tidal cycle. Warwick and Gee (1984) report *M. vivipara* to have a shallow distribution in the sediment and suggest that it is likely to be dispersed by suspension.

In our study, the abundance of *M. chandleri* and *M. obesa* in the water column was disproportionately high in comparison to their sediment abundance. Our results, in conjunction with the earlier studies suggest that the surficial or slightly subsurficial vertical distribution of *Metachromadora* (Table I), their morphology, and their lethargic behavior increased the probability of their suspension. Furthermore, the relatively low abundance of *M. chandleri* and *M. obesa* in the sediments and high abundance in the water, suggests that at least a proportion of the study site water column population may have been derived from other marsh areas. Indeed, a large sand bar, which is a typical habitat for *M. obesa,* is just upstream of the sampling site.

All three species displayed tidal periodicity in water column abundance with lowest numbers during slack water when bottom friction velocity was well below that necessary to erode sediments (Palmer and Gust, 1985; Table IV). This reinforces the conclusion of Palmer and Gust (1985) that nematodes were passively dispersed. Flow dynamics and meiofaunal response in other marsh areas must have followed a pattern similar to that observed above the mudflat because a significant relationship between flow over the mudflat and meiofaunal suspension was found for both locally eroded and advected animals. Current transport is probably a major factor in the dispersal and colonization abilities of *Metachromadora* species.

If nematodes do reach other habitats, these immigrants enhance the diversity of the nematode community in that habitat. For example, the *M. obesa* found in our sediment samples may have been advected from the nearby sandbar. We cannot know whether those specimens are part of a reproducing mudflat population or just immigrants which have managed to survive. Specimens were undamaged and fully stained upon collection. The consistent presence of these nematodes in the water suggests that immigration may occur each tidal cycle. The same processes presumably operate on less abundant species which occur in the surface sediments.

Chromadorita aff. *minima* has only been reported in the original description from the English Channel (Gerlach and Riemann, 1973) and there are minor morphometric and morphological differences between our specimens and the original description. Other species of *Chromadorita* reportedly swim (Jensen, 1981) and occupy both sedimentary and phytal habitats. The ability to survive in the water column would be advantageous to a phytal species which requires a small, patchy, ephemeral (in North Inlet) habitat such as macroalgae.

Ptycholaimellus (= Hypodontolaimus) pandispiculatus is a characteristic species of the surface layers of subtidal sediment (Ott, 1972). It may not be particularly prone to suspension (<0.1% of sediment abundance was suspended here), but because it is so abundant in the sediment (Table II), even if only a small percentage is suspended there can be significant effects on the dispersal of the species. Bell and Sherman (1980) found *P. pandispiculatus* to be the second most abundant species suspended at the sediment-water interface and in the water column above the sediment, concomitant with a high sediment abundance. We found lower numbers in the water, but our samples were taken 17 cm off the bottom, not at the sediment-water interface. We may not have captured specimens near the bottom. Even so, *P. pandispiculatus* was among the most abundant suspended nematode species in this study and also among the first and most abundant species to recolonize the disturbed area of Sherman and Coull (1980). The combined evidence from Bell and Sherman (1980), Sherman and Coull (1980), and our study suggests that the dispersal capabilities, and thereby the colonization abilities of this species are enhanced by advective currents.

Spilophorella paradoxa, a cosmopolitan species, is found on many coasts in the North Atlantic (Gerlach and Riemann, 1973). *Daptonema erectum* has been found all along the eastern coast of the United States (Florida and Georgia—Gerlach and Riemann, 1973; South Carolina—Eskin, 1985; New York—Eskin, pers. obs.). *Viscosia papillata* has been found from Texas to Maryland on the Gulf and Atlantic coasts (Gerlach and Riemann, 1974). All of these species have a wide geographic distribution, which may be a result of their tendency to be suspended, and probably transported by currents. Thus, suspension may play an important role in nematode zoogeographic patterns.

Abundant sediment species such as *Sabatieria pulchra* (G. Schneider, 1906), *Terschellingia longicaudata* De Man, 1907 and *Odontophora setosa* (Allgen, 1926) consistently have been found in the deeper sediment layers (Table I) and this may explain why they were rarely suspended.

Current transport apparently plays a significant role in the dispersal of certain meiobenthic nematodes. Water column dispersal of nematodes depends on their vertical distribution in the sediment. Other considerations include the sediment abundance of a species (*e.g., Ptycholaimellus pandispiculatus*) and their morphology or behavioral characteristics (swimmers *vs.* non-swimmers, lethargic *vs.* active). A corollary to the role of current transport in dispersal is an enhanced ability to reach and thereby colonize new or disturbed habitats (Sherman and Coull, 1980). Diversity may be affected as animals are carried from habitats in which they may be abundant to new habitats where they settle. Even if it is an unsuitable habitat for the colonizers, if they are constantly being deposited, they will be represented in samples. Once meiofauna are in the water column, they are subject to the same hydrodynamic forces and associated interactions with bottom topography as any small planktonic organism (*e.g.,* Eckman, 1979, 1983; Hannan, 1984). Small-scale patchiness, such a conspicuous feature of nematode communities, may also be significantly influenced by current transport (*e.g.,* Hogue and Miller, 1981). These ideas await further testing.

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FLOW-ASSISTED SHELL REOPENING IN SWIMMING SCALLOPS

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ABSTRACT

As a result of superambient pressures at the gape and of subambient pressures lateral to the valves, flow-induced forces augment the action of the hinge ligament in reopening scallop shells between cycles of closing. As the gape angle increases, flow-induced forces increase while the recoil of the hinge ligament decreases. At a gape angle of 13.3° and a speed of 0.5 $\text{m} \cdot \text{s}^{-1}$, the overall hydrodynamically generated opening moment is estimated as 22.3% of that due to ligament elasticity.

INTRODUCTION

A scallop swims by opening and closing its two valves; typically water enters the ventral gape and is expelled through dorsal openings at either side of the hinge, causing the animal to progress ventrally in a somewhat unsteady fashion. A large adductor muscle closes the valves; an elastic ligament is inevitably described as the opener.

An additional agency may be involved in reopening during swimming. Dynamic pressure at the gape will increase the internal pressure, as suggested by Moore and Trueman (1971), and pressures lateral to the valves will decrease due to flow along their convex outer surfaces. In concert, these consequences of motion-induced flow and Bernoulli's principle will create outward transmural pressures. Of principal concern here is the magnitude of the opening moment due to hydrodynamic forces relative to the moment due to elastic recoil of the ligament.

In practice, hydrodynamic forces were assessed by measuring the pressures at a series of loci on each valve and on an artificial mantle installed in an empty shell. Elastic forces were measured by incrementally closing a freshly eviscerated shell using a strain gauge-equipped beam on a manipulator.

MATERIAL AND METHODS

Specimens of *Argopecten irradians* (= *Aequipecten irradians* = *Pecten irradians*) were obtained from Bogue Sound, North Carolina. A single pair of cleaned and dried valves, 62 mm in maximum dorso-ventral length, were used for pressure measurements. For measurements of ligament elasticity, four animals ranging from 62 to 69 mm were maintained in cold seawater for two days. Each shell with ligament was quickly isolated, and measurements were completed at 21°C in ten minutes.

For pressure measurements a shell was prepared as follows. A 1 × 1 cm rectilinear grid of 22 points was projected onto each valve and each point perforated with a 1 mm dental drill (Fig. 1a). A 2.4 mm brass tube extending dorsally from an additional hole in the right valve provided both support and pressure-transmitting conduit. For simulation of a gaped shell, a piece of brass plate was fitted in a position analogous to the teeth in dentures; it was perforated by nine additional 1 mm holes 1 cm apart. The resulting gape angle of 13.3° was an "ordinary" value—the maximum gapes in actively

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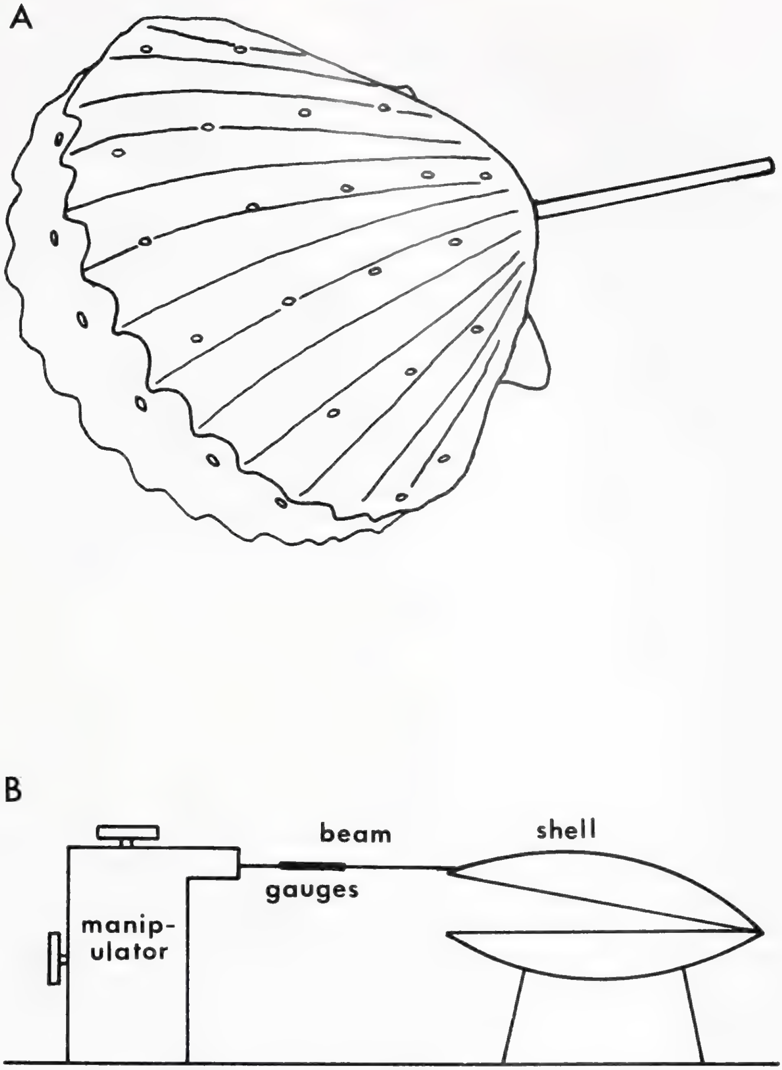

FIGURE 1. (a) Scallop shell with gape insert and pressure transmitting conduit as prepared for pressure measurements in wind tunnel; tracing from photograph. (b) Diagrammatic view of beam with strain gauges mounted on manipulator and arranged to monitor force as valves are moved together.

swimming scallops are around 25° (Patrick Woodbury, pers. comm.). Initially all holes were blocked with melted wax; additional wax joined the two valves with or without the gape insert.

Pressure measurements were made using a multiplier manometer in a large wind tunnel (Tucker and Parrott, 1970); data were expressed as dimensionless pressure coefficients. The use of air instead of water as working fluid is discussed in Vogel (1981); the particulars of calibration of tunnel and multiplier manometer and of conversion to pressure coefficients are given in Vogel and Loudon (1985). Data cited here were obtained at a speed of 7.2 $m \cdot s^{-1}$, equivalent to 0.5 $m \cdot s^{-1}$ in 20°C seawater. For calculation of moments, pressure coefficients were converted to pressures in water by multiplying by the dynamic pressure based on the density of seawater and its equivalent velocity.

Most measurements of pressure difference used as reference a 1 mm static aperture, 10 cm downstream from the leading edge of a flat plate parallel to flow and about 15 cm from the shell. The high pressures for the three most ventral holes in the gape insert were referred instead to the apical orifice of a commercial Pitot-static tube facing upwind, and an additional unit of pressure coefficient was added to the data in compensation. The shell was oriented in the center of the wind tunnel so that the commissure between the valves or the center of the gape was within two degrees of being parallel to flow with the ventral edge directed upwind. Each hole in turn was opened for two determinations of pressure difference made so as to compensate for drift in the manometer (never more than 0.4 Pa or $N \cdot m^{-2}$).

Pressures at holes on the valves were converted to moments about the hinge by multiplying each first by the sagittal (commissural) projection of the area of valve to which each referred and then by the mean, perpendicular distance in the sagittal plane between the hinge-line and that area. Pressures at holes in the gape insert were again multiplied by their corresponding areas; and, assuming that the average of these data acted at the geometrical center of each valve, they were further multiplied by the projected distance in the sagittal plane between the center and the hinge. The different treatment of holes in valves and gape presumes that the soft mantle blocking the gape normally transmits no appreciable force directly to the valves.

To measure the moment due to the ligament the left valve of each eviscerated shell was pressed into a concave block of modelling clay with the commissure between valves horizontal. An aluminum beam, 60 mm × 12.7 mm × 1.27 mm, with foil strain gauges on each face was attached at one end to a manipulator with a worm drive; the other end touched the right valve 1 mm dorsal to the ventral edge (Fig. 1b). The beam was lowered in increments of 1.27 mm with horizontal adjustments as necessary until the valves touched; the distance from the last standard increment was noted. Deflection of the beam itself, maximally 0.16 mm, was ignored. The output and calibration of the strain gauges were treated as described by Vogel and Loudon (1985). The components of these forces normal to the sagittal plane were multiplied by the projected distance in that plane from the hinge to the point of contact between beam and valve.

RESULTS

Table I gives the pressure coefficients for the valves of the closed shell and for the shell with a gape of 13.3°, together with those for the gape insert; the arrangement of data corresponds to the locations of holes shown in Figure 1a. Pressure coefficients may be converted to pascals (Pa) of pressure in air (as actually measured) by multiplication by 31.0 or in 20°C seawater by multiplication by 128.0.

Several items are noteworthy. (1) The most ventral hole in the gape insert gives a pressure coefficient of unity as expected for a stagnation point. (2) Pressures on the ventral five holes of the gape are positive while the four dorsal holes give slightly negative pressures. The latter correspond to only 40% of the overall gape area; thus net flow-induced pressure on the gape increases internal pressure, pushing the valves outward. (3) Pressures on the valves are predominantly negative, pulling the valves outward. Only the ventral-most holes ever give positive pressures; these are greater for the closed shell where the ventral-most holes face more directly into the flow. These latter holes have the greatest lever-arm contributing to their moments but represent

TABLE I

Pressure coefficients for scallop shells

		Closed shell						*Gaped shell*		
		left valve			ventral			left valve		
	+.14	+.08	+.02				−.13	−.06	−.08	
−.23	−.15	−.14	−.13	−.35		−.40	−.22	−.19	−.20	−.33
−.25	−.19	−.18	−.24	−.33		−.30	−.22	−.17	−.20	−.25
−.24	−.20	−.17	−.21	−.27		−.22	−.16	−.13	−.16	−.21
	−.17	−.14	−.18				−.09	−.11	−.13	
		−.11						−.08		
hinge					dorsal					hinge
		−.15						−.04		
	−.17	−.16	−.19				−.09	−.07	−.08	
−.24	−.23	−.16	−.27	−.31		−.20	−.13	−.15	−.11	−.16
−.43	−.19	−.15	−.29	−.39		−.26	−.19	−.20	−.19	−.28
−.24	−.28	−.10	−.31	−.32		−.18	−.18	−.25	−.28	−.31
	+.34	+.24	+.18				+.04	+.05	+.03	
		right valve			ventral			right valve		
ant.				post.		ant.				post.
					gape					
ant.	−.30	−.16	+.13	+.81	+1.00	+.82	+.25	−.13	−.28	post.

only 12% of the overall shell area. (4) Pressures are most negative for the more anterior and posterior holes. Possibly the separation of flow described by Thorburn and Gruffydd (1979) is less severe in these regions; certainly the less negative pressures for the middle dorso-ventral rows of holes are consistant with substantial separation and relatively high drag (Vogel, 1981). (5) Much of the scatter of the data probably comes from the variability in the location of holes relative to the conspicuous ribbing of the valves.

For the closed shell the moment on the right valve (computed for seawater) was 0.00178 Nm; for the left valve it was 0.00183 Nm, giving an average moment of 0.00180 Nm tending to open the shell. (Using the average rather than the sum of the moments reflects the advice of engineers K. Pete Arges and Gale H. Buzzard.) For the gaped shell the moment on the right valve (again computed for seawater) was 0.00174 Nm; for the left valve it was 0.00230 Nm, giving an average moment of 0.00202 Nm. (The apparent lift due to the difference between these figures probably reflects little more than sensitivity of the two moments to the orientation of the shell.) The moment due to pressures at the gape was 0.00126 Nm. Combining the moment due to pressure at the gape with that for each valve and then averaging for the two valves gives an overall moment tending to open the shell of 0.00328 Nm.

Figure 2 gives the behavior of the hinge ligament, data obtained by incrementally moving the valves together while monitoring the force required to do so. No correction for size of animal has been applied since within the narrow size range examined no regular variation was apparent. In view of the minimal hysteresis (in scallops, at least) found by Trueman (1953) and Kahler *et al.* (1976), separate data was not obtained for incremental opening of the shells; the slight curvature of the plot is similar to their data. Clearly, the ligament is most effective at generating force when the shell is closed and becomes rapidly less effective as the gape increases—for closed shells the extrap-

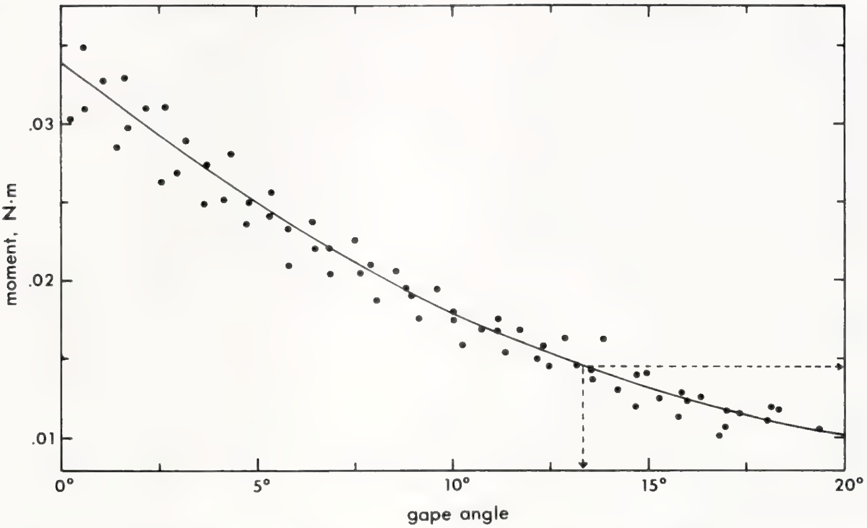

FIGURE 2. Moment required to prevent valves from moving apart as a function of gape angle. Most of the scatter reflects differences among the four specimens. The curve is eye-fitted and presumes no *a priori* relationship. Dashed line indicates moment at the 13.3° gape used in pressure measurements.

olated average moment is 0.0340 Nm, while for shells with the 13.3° gape used for pressure measurements the average moment is 0.0147 Nm.

For the closed shell, the overall hydrodynamically generated moment is 0.0018/0.034 or 5.3% of the elastically generated moment. For the gaped shell, the overall hydrodynamically generated moment is 0.00328/0.0147 or 22.3% of the elastically generated moment.

DISCUSSION

Neither the imprecision nor the systematic errors in determination of pressures or forces have been specifically addressed in presentation of the data—the overall uncertainty in pressure measurements was less than 0.3 Pa (in air) or less than a pressure coefficient of 0.01; moments derived from force measurements had an overall uncertainty of 0.001 Nm before averaging among the four specimens. In practice these uncertainties must have been much smaller than those which were intrinsic to the underlying experimental design. Thus (1) in life the gape is only slightly occluded by the soft tissues of the mantle by contrast with the complete occlusion of the perforated brass insert. Water entering the scallop will relieve the pressure at the gape; this might reduce the overall hydrodynamic moment by as much as 20% for a moderate value of gape such as used here, if rather less for near minimal and maximal gapes. And (2) all pressure measurements were carried out in steady flow whereas the normal motion of a scallop shell is most unsteady (*sensu* Daniel, 1984), with about three cycles of opening and closing each second (Alexander, 1966). For a rapidly opening shell, the severe separation of flow noted earlier is less likely, so in practice the moments due to reduced pressures lateral to the valves may be as much as 30% higher. In view of these general uncertainties the present study was limited to demonstration of the probable existence and significance of the basic phenomenon and did not consider effects of different angles of the commissure with the oncoming flow, other gape angles, and other flow speeds.

Perhaps the major uncertainty in interpreting the present data is that of the swimming speed of this and other scallops since the pressures generated by flow are nearly proportional to the square of velocity. The choice of $0.5 \text{ m} \cdot \text{s}^{-1}$ was a compromise. Had, for example, $0.6 \text{ m} \cdot \text{s}^{-1}$ been chosen instead, the flow-induced moment relative to the elastic moment would have been about 32% instead of 22% for the gaped shell. Swimming speeds for *Chlamys opercularis* of about the same size as the present scallops have been estimated as above $0.3 \text{ m} \cdot \text{s}^{-1}$ (Moore and Trueman, 1971); $0.34 \text{ m} \cdot \text{s}^{-1}$, with a comment noting probable underestimation (Thorburn and Gruffydd, 1979); and above $0.35 \text{ m} \cdot \text{s}^{-1}$ (Gruffydd, 1976). For the particularly light-shelled, flat-valved *Amusium pleuronectes,* Morton (1980) reported a speed of $0.73 \text{ m} \cdot \text{s}^{-1}$ in an animal only slightly larger (70 mm high) than the present ones. All of these data are from measurements in laboratory tanks. For the larger *Placopecten magellanicus,* Caddy (1968) reported a figure of $0.68 \text{ m} \cdot \text{s}^{-1}$ based on time and distance from take-off to landing of animals in nature avoiding a scallop drag: speeds during horizontal motion must have reached at least $0.8 \text{ m} \cdot \text{s}^{-1}$. A comparative study of the kinematics of swimming in scallops is now underway (Patrick Woodbury, pers. comm.).

The present data for moments due to the hinge ligament are reasonably close to those previously reported. Trueman's (1953) figure for the moment at closure for *C. opercularis* is about 20% higher. Kahler *et al.* (1976) cite lower figures for *A. irradians* than those here, but the differences are easily accounted for if their animals were about 40% shorter.

The contribution of flow-induced pressure differences to reopening is likely to be less than that of the elasticity of the hinge ligament under all circumstances. Nonetheless, these hydrodynamic forces may be of substantial functional significance due to the particular circumstances under which they are maximal. Thus the strong dependence of pressure differences on flow speed means that reopening will be most assisted at the highest swimming speeds; indeed the present phenomenon may be viewed as a mechanism for deriving some functional benefit from the drag which slows them almost to a stop between cycles of valve closing (Patrick Woodbury, pers. comm.). Moreoever, the increase in the flow-induced moment with increasing gape means that the hydrodynamic effect will be greatest when the moment due to elasticity of the hinge ligament is least; for an animal in rapid motion, achieving a slightly wider gape between closings as well as slightly more rapid reopening may be no minor matter.

ACKNOWLEDGMENTS

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PATTERN OF CELLULAR ALLOREACTIVITY OF THE SOLITARY ASCIDIAN, *HALOCYNTHIA RORETZI*, IN RELATION TO GENETIC CONTROL

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ABSTRACT

The pattern of cellular alloreactivity in the solitary ascidian, *Halocynthia roretzi* (Drashe), was examined using 435 combinations of 30 individuals. A high level of polymorphism was revealed and no two individuals had identical histocompatibility even though they were sampled in a relatively small area of Mutsu Bay.

In *H. roretzi,* the allogeneic reaction seems to be triggered by an absence of common self markers, as in the colony fusibility of *B. primigenus,* rather than by a specific reaction to non-self markers as in higher vertebrates. We analyzed the allelic compositions of 30 animals assuming that a non-reactive pair share an allele. Since at most four "alleles" are carried by each individual, the solitary ascidian most probably has two genes that control alloreactivity.

INTRODUCTION

Recent studies indicate that the ability to distinguish self and non-self components is one of the intrinsic characteristics of living organisms (Hildeman, 1974; Cooper, 1976; Coombe *et al.*, 1984). This ability is widespread throughout the biosphere from lower organisms, such as the slime mold (Carlile, 1972) and sponge (Hildeman, 1979; Curtis *et al.*, 1982), to higher vertebrates such as the mammals (Klein, 1975; Götze, 1977). Whether there is a homologous relationship among different animals, especially between invertebrates and higher vertebrates, remains to be determined.

Oka and his colleagues have shown that the hermaphroditic colonial ascidian *Botryllus primigenus* Oka (Stylidae) has a single multiallelic histocompatibility (H) gene (Oka, 1970; Tanaka and Watanabe, 1973). Ascidians represent a class of Tunicata, the most primitive living branch of the phylum Chordata (Berrill, 1955). It is therefore possible that the major histocompatibility complex (MHC) of the vertebrates has evolved from a gene not very different from the H gene of *B. primigenus* (Scofield *et al.*, 1982). This gene controls two types of cell recognition systems: intercolonial compatibility at the somatic cell level and fertilization at the gamete cell level. When cut surfaces of two colonies are brought into contact, they either fuse or reject each other. Fusion ensues when the two colonies share an allele of the H gene. If the colonies do not share an allele, mutual rejection ("nonfusion reaction") occurs. Thus, colony fusibility is determined by the presence or absence of self (*i.e.,* shared) rather than non-self (*i.e.,* not shared) H gene controlled markers, a crucial point discussed previously by several authors (Burnet, 1971, 1976; Mäkelä *et al.*, 1976; Laffery and Woolnough, 1977). Fertilization takes place if the single allele carried by spermatozoa is not shared by the egg donor, presumably because self recognition between haploid spermatozoa and the diploid cell-derived chorion of the egg prevents fertilization (Oka,

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1970). All individuals of natural *B. primigenus* populations are, therefore, heterozygous for the H gene, suggesting that the gene has evolved as a genetic mechanism for obligatory outbreeding, like the *S* genes of flowering plants (Lewis, 1976; Clarke and Knox, 1978). The remarkably high frequency of *H-2* heterozygosity in wild mice (Duncan *et al.,* 1979a) may indicate that a similar function is retained by the murine MHC and/or other related genes on the same chromosome (Hammerberg and Klein, 1975).

An *in vitro* cellular counterpart of the intercolonial nonfusion reaction has recently been described for the solitary (as opposed to colonial) ascidians (Fuke, 1980). The contact reaction, as the *in vitro* reaction was termed, requires direct contact between xenogeneic or allogeneic blood cells, is reciprocal in that two cells in contact lyse each other, and is a rapid reaction that is complete within a few minutes. The allogeneic reactions were observed between blood cells from some but not all donor combinations. In the present communication, we report a genetic analysis of alloreactivity in *Halocynthia roretzi* (Drashe), a hermaphroditic solitary ascidian of the family Pyuridae.

MATERIALS AND METHODS

Three variant types of *H. roretzi* (Type A, B, C) inhabit the coast of northern Japan (Numakunai and Hoshino, 1973, 1974). Type A individuals, which could be readily distinguished from other types by morphological criteria, were used for this study. They were randomly collected at 10 to 20 meters by SCUBA diving along approximately 120 meters of the shoreline of Futagojima Island in Mutsu Bay, Aomori.

The "contact reactions" were tested as follows: blood cells were collected from the mantle-test interspace by withdrawing body fluid. Initially, the blood cells from one animal were cultured in a small glass chamber and vitally stained. The suspension of the cells from a second animal was introduced into the chamber. Contact reactions began immediately and were observed under phase contrast microscopy. Typically, after two cells from different individuals came into contact, one cell moved around the other as if it had been scouting for the other. In a short time, the former stopped and pressed itself to the latter. Within seconds, one cell was lysed, followed immediately by lysis of the other. To determine whether a reaction occurred the cells were observed one hour after mixing. A full account of experimental procedures and details of the contact reaction were given in a previous paper (Fuke, 1980).

All 435 different combinations of the 30 animals were tested at least twice. All experiments were carried out at aquarium facilities of the Biological Station, Asamushi, Aomori.

RESULTS

The results of the mixed culture tests involving 435 combinations, are shown in Table I. No individuals reacted with all of the other 29 animals. The minimum number of non-reactive combinations found in each of 30 individuals was one (No. 20 and 27), while the maximum number was 9 (No. 18). Thus, the frequency of nonreactive allogeneic combinations for each animal ranged from 3.4% to 31% and averaged 15.4%, which was similar to the frequency observed in other types of *H. roretzi* (Fuke and Numakunai, 1982).

Absence of alloreactivity between two individual mice indicates lack of disparity in their histocompatibility antigens. In *H. roretzi,* however, absence of alloreactivity between two animals does not necessarily mean that they are identical in their histocompatibility. For example, animals No. 1 and No. 3 did not react with each other and shared parallel reactivity towards No. 4 and No. 29, yet differed in their reactivity to No. 7, No. 10, etc.

TABLE I

*Reciprocal alloreactivity of 30 individuals**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	−		−	−			−			−									−										−	
2		−													−											−	−			
3	−		−	−				−	−								−												−	
4	−		−	−				−				−						−	−										−	−
5					−						−			−							−									−
6						−							−			−								−				−		
7	−						−			−							−													
8			−	−				−	−			−			−		−	−												−
9			−					−	−				−		−		−	−					−							
10	−						−			−																				
11					−						−		−								−				−					−
12				−				−				−						−												−
13						−			−		−		−										−	−	−					
14					−									−							−			−						
15		−						−	−						−		−	−								−				
16						−										−			−					−				−		
17			−				−	−	−						−		−	−												
18				−				−	−			−			−		−	−				−		−						−
19	−			−												−			−										−	
20																				−								−		
21					−						−			−							−									
22																		−				−	−							
23									−				−									−	−							
24						−							−	−		−		−						−				−		
25											−		−												−				−	
26		−													−											−				
27		−																									−			
28						−										−				−				−				−		
29	−		−	−															−						−				−	
30				−	−			−			−	−						−												−

* A minus (−) sign indicates absence of reactivity; unmarked combinations were reactive.

Among the 30 animals, there were some pairs or groups which had similar, but not identical, alloreactivity. For example, individuals No. 12 and No. 30 did not react with each other and showed the same reactivity to No. 4, No. 8, and No. 18 but differed in reactivity to No. 5 and No. 11.

DISCUSSION

In a previous study (Fuke, 1980), two animals, behaving as if they were "syngeneic," were found in a smaller panel employing 16 individuals. The present data, however, obtained from a larger number of animals show that none of the mutually nonreactive animals have the same pattern of reactivity against the rest of the animals and suggest that there are no syngeneic pairs in nature.

The results can be explained if we assume that the reaction took place only when two individuals did not share an allele of an H gene or genes. This simple genetic model is identical with the one that accounts for colony fusibility of *B. primigenus*. It is possible that more complex genetic models also explain the data, but the Occam's choice would be the one presented above. According to this model, a nonreactive pair must share at least one allele. We now assume that such a pair shares only one allele. An arbitrary letter was assigned to each of the alleles identified by histocompatible groups of two or more mutually nonreactive animals. This operation is illustrated in Figure 1 for the first seven alleles, *a* through *g*. The allelic compositions of the 30 animals thus obtained are listed in Table II. Since as many as four "alleles" are carried by some individuals, the solitary ascidian must have two or more H genes that control alloreactivity. As implied by the genetic model on which this analysis is based, these H genes are functionally equivalent, at least as far as their control of alloreactivity is concerned.

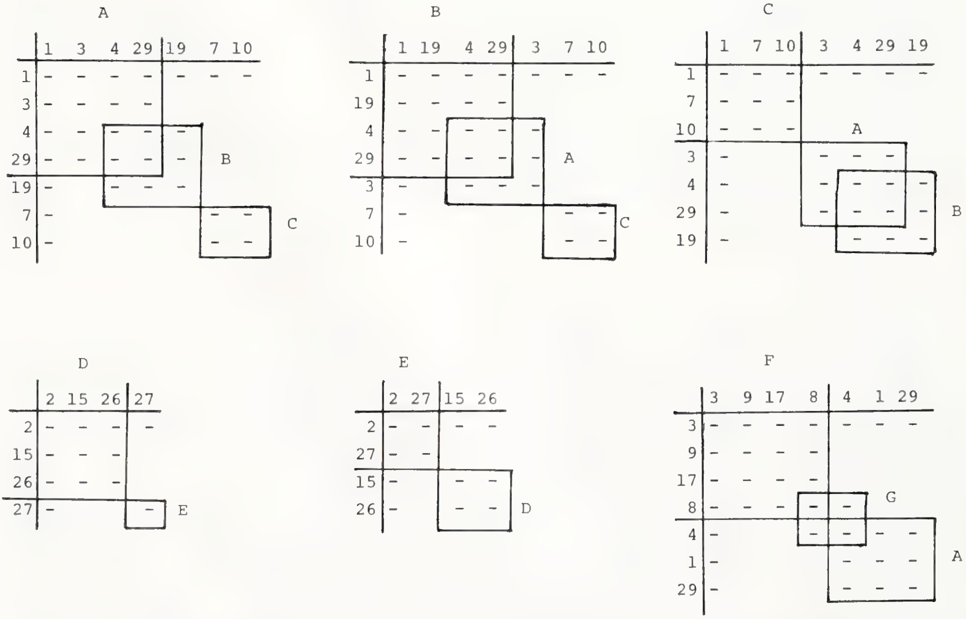

FIGURE 1. Illustration of histocompatibility group analysis. The group *A* through *G* identify alleles *a* through *g*, respectively.

TABLE II

Allelic compositions of the H genes in individuals

Animal No.	Allelic composition																								No. of alleles
1	a	b	c																						3
2				d	e																				2
3	a					f	g																		3
4	a	b					g	h																	4
5									i	j	k														3
6												l	m												2
7			c											n											2
8						f	g	h							o										4
9						f									o	p									3
10			c																						1
11									i	j							q								3
12								h																	1
13												l				p	q								3
14											k							r							2
15				d											o										2
16													m						s						2
17						f								n	o										3
18								h							o					t	u				4
19		b																	s						2
20																						v			1
21									i		k														2
22																				t			w		2
23																p							w		2
24												l	m					r			u				4
25																	q							x	2
26				d																					1
27					e																				1
28													m									v			2
29	a	b																						x	3
30								h		j															2

Assuming that the solitary ascidian has only two H loci and that, unlike the *B. primigenus* H gene, one or both of the loci can be homozygous, we find the total minimum number of alleles represented in the panel to be 29 (those organisms which were assigned only one or two alleles were assumed to be homozygous for both loci; those with three alleles were considered to have one homozygous and one heterozygous locus). On the other hand, if none of the organisms tested were homozygous at either of the loci, 73 would be the maximum number of alleles represented by the panel. The average frequency of alleles in these cases would be 6.9 and 2.7, respectively (in the latter case, for example, the frequency of the *h* or *o* allele is 5/60, that of *a, b, f,* or *m,* is 4/60, etc., and 49 unnamed alleles appear only once with a frequency of 1/60). Since the organisms of this panel were sampled in a relatively small area of a bay, even the lower value could be an overestimate. For comparison, a similar analysis was based on published data regarding the *B. primigenus* H gene. Since all naturally occurring colonies are heterozygous for the H gene, as pointed out earlier, Tanaka and Watanabe's data (1973) indicate that 80 to 83 alleles were represented by their panel of 45 randomly collected colonies, yielding an average alleleic frequency of 1.2%. As 11 out of 990 different pairs from 45 colonies were nonreactive (*i.e.*, sharing an allele), the total number of alleles of the H gene should be several hundred according to Bateman's methods (1947) of estimation. Judging from the average allelic frequencies, the extent of H gene polymorphism per locus seems somewhat higher in the colonial than in the solitary species. However, the existence of two H loci in the latter organism should confer as much or even more overall genetic diversity as the single locus does for the colonial species. Interestingly, the polymorphism of the mouse MHC (the H-2) class I loci is of similar magnitude (Duncan *et al.*, 1979b).

B. primigenus and *H. roretzi* belong to two related families of the same suborder (Millar, 1966). Furthermore, according to Millar (1966), the family Pyuridae to which *H. roretzi* belongs may have been derived from a primitive stock of Styelidae, which includes the genus *Botryllus*. If Millar's view is correct, it is possible that the redundant H genes of the solitary ascidian arose by a recent duplication and that the H genes of these organisms represent two successive stages in a monophyletic line of MHC evolution.

In a previous experiment, fertilization was observed between gametes from alloreactive as well as nonreactive donors (Fuke, 1980). The results are consistent with the haploid-diploid compatibility postulated for *B. primigenus* fertilization, but do not exclude a number of other possibilities that include, for instance, total lack of H gene control over fertilization. Therefore, it remains to be seen whether or how these H genes control the reproduction of *H. roretzi*. Experiments with "mosaic eggs" have recently revealed that the chorion of the eggs has specific ability to recognize self components of sperm (Fuke, 1983). Immunochemical studies of the coelomocyte's membranes and the chorion of unfertilized eggs are in progress to determine whether there are common factors responsible for recognition of self *versus* non-self.

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GENETIC BASIS OF SHELL VARIATION IN *THAIS EMARGINATA* (PROSOBRANCHIA, MURICACEA). I. BANDING IN POPULATIONS FROM VANCOUVER ISLAND

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ABSTRACT

Thais (or *Nucella*) *emarginata*, a rocky intertidal gastropod inhabiting the Pacific coast of North America, is polymorphic for shell banding in nearly all habitats in which it is found. Two generations of breeding revealed that this polymorphism has a simple genetic basis. Banding of the outer shell was inherited as if controlled by a single autosomal locus (OB) with two alleles (OB^B = banded and OB^U = unbanded), with banding dominant. Banding also assorted independently of the major shell color locus (OC), thus reinforcing an earlier conclusion that shell traits in this species are not tightly linked in a 'super gene' as in many terrestrial pulmonates. The clarity of banding, however, was found to depend on other genes or alleles influencing pigment intensity; individuals carrying these alleles at the banding or other loci exhibited much less pronounced banding or in some cases a complete loss of pigment in the outer shell. Most commonly, spiral bands appear as regularly spaced lines of pigment set against a largely unpigmented background (= white to pale grey ground color), but bands may also occur against other ground colors. Mechanistically, however, banding appears to result from regularly spaced zones of suppressed pigmentation in the outer shell. Preliminary distributional data revealed that the frequency of banded individuals in field populations increased with increasing wave exposure; however, the adaptive value of this polymorphism is not clear at present.

INTRODUCTION

Many species of marine, shallow-water, prosobranch gastropods exhibit intraspecific variation in shell pigmentation (Kincaid, 1957, 1964; Spight, 1976; Clarke, 1978). The rather conspicuous variation in some species has prompted numerous studies of the correlation between morph-frequencies and environmental conditions (Colton, 1922; Pelseneer, 1935; Moore, 1936; Fischer-Piette *et al.,* 1963; Daguzan, 1968; Safriel, 1969; Berry and Crothers, 1974; Heller, 1975; Pettitt, 1975; Hoagland, 1977; Osborne, 1977; Davis, 1980; Bowman, 1981; Reimchen, 1981; Smith, 1981). Surprisingly few have examined experimentally the adaptive value of variation in shell pigmentation (Giesel, 1970; Reimchen, 1979; see also Mercurio *et al.,* 1985). In addition, and in contrast to the situation for aquatic and terrestrial pulmonates (reviewed by Murray, 1975), little is known about the genetic basis of this variation (but see Cole, 1975; Palmer, 1984a). I report here the results from two generations of breeding, including backcrosses: that variation in shell banding in populations of the rocky intertidal gastropod *Thais* (or *Nucella*) *emarginata* is controlled predominantly by a pair of alleles at a single autosomal locus. Knowledge of the genetic basis of shell banding

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variation now sets the stage for an analysis of the factors which maintain this variation as an apparently stable polymorphism in natural populations.

Thais emarginata is a common and well-studied inhabitant of the middle and upper intertidal of rocky shores (Emlen, 1966; Spight and Emlen, 1976; Kitching, 1976; Davis, 1980; Morris *et al.,* 1980; Palmer, 1983, 1984a, b). It has one of the broadest geographic ranges along the Pacific coast of North America of all intertidal gastropods (Palmer, 1984a), and it is an important predator of barnacles and mussels (Palmer 1983, 1984b). Individuals of *T. emarginata* also exhibit a broad range of variation in shell color, banding, sculpture, thickness, and shape (Kincaid, 1964; Emlen, 1966; Abbott, 1974; Kitching, 1976; Davis, 1980; Morris *et al.,* 1980; Palmer 1984a, 1985). By virtue of their direct development (Lyons and Spight, 1973) and the fact that newly hatched snails will feed readily on very small barnacles, *Thais emarginata* may be bred and raised in the laboratory without difficulty (Palmer, 1984a).

MATERIALS AND METHODS

Immature specimens of *Thais emarginata* (<15 mm in nearly all cases) were collected from Wizard Island (48°51′N, 125°09′W), in Barkley Sound near the Bamfield Marine Station, on the west coast of Vancouver Island, British Columbia (Canada). Individuals were sexed according to relative penis size (see also Hargis, 1957); single pairs were placed in separate cages, made from plastic freezer containers whose sides had been replaced with VEXAR® plastic mesh, and were provided with one of their preferred prey (the barnacle, *Balanus glandula;* Palmer, 1984b). Cages were checked at approximately two-week intervals to monitor growth to maturity and egg-capsule production. When laying was completed, each clutch was transferred to a separate, small envelope of plastic window screening with VELCRO® closures where it was held for approximately 60 days while the embryos developed. These envelopes permitted greater water exchange than cages in which hatchlings were raised and resulted in a higher hatching success. Prior to hatching, the capsules were transferred to one liter freezer containers whose sides had been replaced with 500 μm NITEX® screening and provided with stones covered with very young (<3 mm basal diameter) barnacles (primarily *Balanus glandula* or *Chthamalus dalli*). Stones were checked at two week intervals and were replaced as barnacles were eaten; when the hatchlings had reached >3 mm shell length, they were transferred to larger freezer containers with sides of plastic window screening. Once larger than about 5 mm, juvenile *Thais emarginata* were capable of eating adult *B. glandula* (8–10 mm basal diameter). Beyond this size, stones with fresh barnacles were provided at approximately 3 week intervals.

Egg-capsules produced by crosses initiated with mature females (*e.g.,* backcrosses of offspring to parents) were saved only after 6 months had elapsed from the time of first pairing. Few if any sperm remain from previous matings after this time (Palmer, in prep.).

The labelling convention used below encodes several pieces of information (general form = year—lineage number—generation—cross identifier):

Year—year in which the lineage (a unique collection of genes) was initiated.

Lineage number—the number of the lineage initiated in that year.

Generation—the laboratory generation of the offspring to be produced (when referring to a pair of parents) or of the individuals themselves (when referring to a clutch). The prefix B indicates a backcross to a parent or grandparent. For first-generation crosses, a suffix letter (*e.g.,* A, B, etc.) identifies a particular F1 clutch.

Cross identifier (3 parts; not present for first generation crosses)—
  Letter—for bookkeeping (if present).
  Digit(s)—identifies the parents of a cross established for a given generation of a given lineage (the prefix M or F indicates a backcross to either a male or female parent).
  Letter(s)—identifies a clutch or clutches produced by a given pair of parents (labelled sequentially starting with A for each pair of parents).

The frequency of banded individuals in field populations was determined from samples of snails from four different sites: a site with relatively low wave action (NW side of Wizard Island), a site with intermediate wave action (N end of Prasiola Point—48°48′N, 125°10′W), and two very exposed sites (SW tip of Cape Beale—48°47′N, 125°13′W, and SW tip of Cree Island—48°51′N, 125°20′W). At both Wizard Island and Cape Beale, two or more samples were taken from different microhabitat types: either predominantly from among mussels or predominantly from among barnacles. To ensure an unbiased sample, I collected all snails encountered within a given microhabitat type until approximately 100 individuals had been collected. Snails having badly eroded shells were not counted. Within mussel beds, only visible snails were collected (*i.e.*, the mussel bed was not disturbed to locate snails in the interstices).

Wave exposure indices were obtained from Craik (1980), who used weight lost by swinging cement blocks anchored to the bottom as a relative measure of wave activity.

Except for the field samples, Chi-square values presented below were calculated from a comparison of observed frequencies, corrected for continuity (Sokal and Rohlf, 1981, p. 710), to the expected Mendelian frequencies, assuming banding was controlled by a single, autosomal locus with two alleles.

RESULTS

Although variation did exist in the clarity with which banding was expressed, banded individuals could be distinguished readily from unbanded ones (Fig. 1). The inheritance of banding was determined primarily from the genealogy of two lineages, 80-17 and 80-18 (Table I). The parents of 80-17-1A (banded male × unbanded female) produced approximately equal numbers of banded and unbanded offspring (25:29) whereas the unbanded parents of 80-18-1A produced only unbanded progeny. Crosses between unbanded, F1 individuals from these two lineages yielded only unbanded offspring (80-17-2-A4A, -A5A, -A8A, 80-18-2-pool, and 81-79-1A). A nearly 1:1 ratio of phenotypes resulted from crosses between banded and unbanded individuals, regardless of which parent was banded: 260:288 banded:unbanded progeny when the male was banded ($\chi^2 = 1.33$, $P = 0.25$; pooled from clutches 80-17-2-A1A, -A2A,B, -A3A, -A10A, -A12A, -A13A, -A16A, 81-77-1A, and 81-78-1A) and 123:121 banded: unbanded offspring when the female was banded ($\chi^2 = 0.004$, $P = 0.95$; pooled from clutches 80-17-2-A9A,B, -A11A,B, and -A15A). In addition, crosses between two banded F1 individuals yielded close to the predicted 3:1 ratio of banded to unbanded offspring (141:42, $\chi^2 = 0.31$, $P = 0.58$; pooled from clutches 80-17-2-A6A and -A7A). Backcrosses with unbanded (homozygous recessive) F1 offspring confirmed that the banded, male parent of 80-17-1A was heterozygous (80-17-B2-MA), and that the remaining three unbanded parents in both lineages were homozygous recessive (80-17-B2-FD; 80-18-B2-MA, and -FE). Finally, backcrosses between unbanded F1 individuals and unbanded, field-collected individuals yielded only unbanded offspring (81-80-1A, 81-81-1A, 81-82-1B). These patterns were consistent with a genetic model incorporating a single, autosomal locus (OB for outer shell banding) having two alleles: OB^B, a

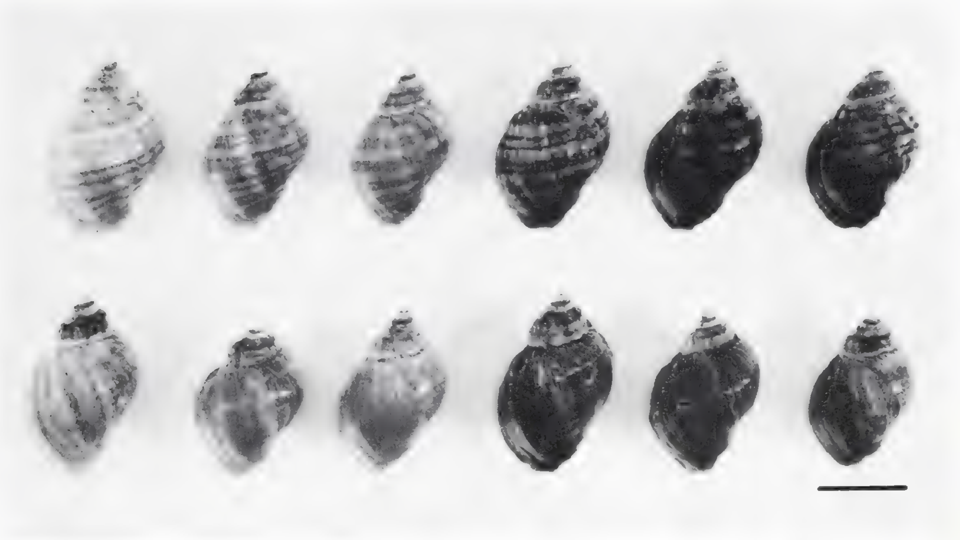

FIGURE 1. Shell banding dimorphism in *Thais* (or *Nucella*) *emarginata* (Deshayes, 1839). Banding phenotypes from clutch 80-17-B2-MA (backcross of unbanded F1 female to original banded male parent; actual phenotype frequencies in Table I). The three left-most individuals in each row are predominantly orange, the three right-most, black. All snails in these clutches were raised from egg capsules deposited in the lab; only the three largest individuals of each phenotype combination were photographed. Approximate age of snails = 9 mos. Scale bar = 10 mm.

dominant allele causing outer shell pigment to be localized in spiral bands, and OB^U, a recessive allele resulting in uniformly pigmented shells.

Crosses in which one parent was heterozygous at both the banding (OB^B/OB^U) and the primary outer shell color locus (OC^{OR}/OC^{BL}), while the other parent was homozygous recessive at both loci (OB^U/OB^U, OC^{BL}/OC^{BL}) revealed that alleles at the banding locus assorted independently from those at the color locus [Group I, Table II; in populations from Barkley Sound the orange, outer shell color allele (OC^{OR}) is usually, but not always dominant to the black allele (OC^{BL}) (Palmer, unpub.)]. Differential survival of phenotypes was suggested by the results of Table II since unbanded, black offspring (OB^U/OB^U, OC^{BL}/OC^{BL}) were not only the most frequent in crosses of Group I, where linkage was tested, but also in the crosses of Group II, where no linkage would be detectable since one parent was heterozygous for banding (OB^B/OB^U), while the other was heterozygous for color (OC^{OR}/OC^{BL}). However, the overall deviation from expected of both groups pooled was not significant ($P = 0.27$, 'Pooled', Table II).

The F1 phenotypes in clutch 82-52-1A (Table I), although consistent with the proposed Mendelian model, reveal the further complexity that the intensity of banding was influenced by other alleles or loci. In this cross, a predominantly unpigmented, essentially unbanded male (light grey shell with pale tan mottling and a white lip), was crossed with a yellow-orange, strongly banded female, yielding six fairly discrete phenotypes (Fig. 2a, b; Table III). Two strongly pigmented morphs (orange and black) exhibited strong banding [two leftmost individuals of rows 3 and 4 respectively, Fig. 2a, b; except for one unbanded black individual (Table III) which, because of its shell shape, was probably a contaminant from another cross or was introduced accidentally with barnacles from the field, a rare but nearly unavoidable problem given the large

TABLE I

Sex, origin, phenotypes, and inferred genotypes of parents and offspring of crosses with Thais emarginata[1]

	Parents				Progeny				
Clutch label	Sex	Source	Pheno.	Genotype	Sex	Banded N	Unband N	χ^2	*P*
F1									
80-17-1A	m	Wizard Is.	banded	$\mathrm{OB^B/OB^U}$	m	15	12	1.48	0.69
	f	Wizard Is.	unband	$\mathrm{OB^U/OB^U}$	f	10	17		
80-18-1A	m	Wizard Is.	unband	$\mathrm{OB^U/OB^U}$	m	—	10	1.33	0.25
	f	Wizard Is.	unband	$\mathrm{OB^U/OB^U}$	f	—	17		
82-52-1A	m	Wizard Is.	unband[2]	?[2]	—	44	39	0.19	0.66
	f	Wizard Is.	banded	$\mathrm{OB^B/OB^U}$, $\mathrm{OB^B/OB^B}$?					
F2									
80-17-2-A1A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	36	37	0.00	1.00
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A2A,B	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	58	45	1.40	0.24
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A3A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	28	34	0.40	0.53
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A4A	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	—	—	32	—	—
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A5A	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	—	—	43	—	—
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A6A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	67	20	0.10	0.75
	f	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$					
80-17-2-A7A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	74	22	0.13	0.72
	f	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$					
80-17-2-A8A	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	m	—	17	0.13	0.72
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	f	—	14		
80-17-2-A9A, B	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	—	77	76	0.00	1.00
	f	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$					
80-17-2-A10A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	19	30	2.04	0.15
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A11A, B	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	—	20	21	0.00	1.00
	f	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$					
80-17-2-A12A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	14	26	3.03	0.08
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A13A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	40	41	0.00	1.00
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-17-2-A15A	m	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$	—	26	25	0.00	1.00
	f	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$					
80-17-2-A16A	m	prog 80-17-1	banded	$\mathrm{OB^B/OB^U}$	—	6	9	0.27	0.61
	f	prog 80-17-1	unband	$\mathrm{OB^U/OB^U}$					
80-18-2-pool[3]	m	prog 80-18-1	unband	$\mathrm{OB^U/OB^U}$	—	—	406[3]	—	—
	f	prog 80-18-1	unband	$\mathrm{OB^U/OB^U}$					

TABLE I (*Continued*)

	Parents				Progeny				
Clutch label	Sex	Source	Pheno.	Genotype	Sex	Banded N	Unband N	χ^2	*P*
F2									
81-77-1A	m	prog 80-17-1	banded	OB^B/OB^U	—	28	35	0.57	0.45
	f	prog 80-18-1	unband	OB^U/OB^U					
81-78-1A	m	prog 80-17-1	banded	OB^B/OB^U	—	31	35	0.14	0.71
	f	prog 80-18-1	unband	OB^U/OB^U					
81-79-1A	m	prog 80-17-1	unband	OB^U/OB^U	—	—	50	—	—
	f	prog 80-18-1	unband	OB^U/OB^U					
Backcrosses									
80-17-B2-MA	m	par 80-17-1	banded	OB^B/OB^U	—	32	28	0.15	0.70
	f	prog 80-17-1	unband	OB^U/OB^U					
80-17-B2-FD	m	prog 80-17-1	unband	OB^U/OB^U	—	1[4]	59	—	—
	f	par 80-17-1	unband	OB^U/OB^U					
80-18-B2-MA	m	par 80-18-1	unband	OB^U/OB^U	—	—	53	—	—
	f	prog 80-18-1	unband	OB^U/OB^U					
80-18-B2-FE	m	prog 80-18-1	unband	OB^U/OB^U	—	—	47	—	—
	f	par 80-18-1	unband	OB^U/OB^U					
81-80-1A	m	Wizard Is.	unband[2]	OB^U/OB^U[2]	—	—	125	—	—
	f	prog 80-18-1	unband	OB^U/OB^U					
81-81-1A	m	Wizard Is.	unband[2]	OB^U/OB^U[2]	—	—	38	—	—
	f	prog 80-17-1	unband	OB^U/OB^U					
81-82-1B	m	Wizard Is.	unband[5]	OB^U/OB^U[5]	—	—	28	—	—
	f	prog 80-17-1	unband	OB^U/OB^U					

[1] Entries under Source: Wizard Is. = collected as immature animals from the field, prog = progeny of specified cross, par = original parent of specified cross. Pheno. = observed phenotype of parents or offspring. Genotype = inferred genotype(s) of parents (OB^B = dominant allele for 'banded', OB^U = recessive allele for 'unbanded'). χ^2 = Chi-square value from comparing observed phenotype frequencies with those expected from a two allele polymorphism at a single autosomal locus. *P* = exact probability.

[2] Parent was unpigminted, thus the parental genotype could only be inferred from the offspring phenotype frequencies.

[3] Pooled offspring from 12 separate crosses.

[4] Probably an accidental contaminant (see text).

[5] Parent was largely unpigmented (with very faint tan mottling), see footnote 2.

number of crosses and their frequency of handling]. Two morphs with pale pigmentation (pale orange and pale brown) exhibited faint banding that increased in intensity towards the more anterior and more posterior edges of the whorl (two rightmost individuals of rows 1 and 2 respectively, Fig. 2a, b). Finally, two morphs were unbanded and their outer shell coloration intergraded to some extent; both were predominantly unpigmented (very pale grey, although some exhibited very pale yellow mottling), but one morph had a white columella and the other clearly had a purple columella (two rightmost individuals of rows 3 and 4 respectively, Fig. 2a, b).

The results of the field surveys for banding phenotype frequencies revealed a pronounced increase in the frequency of banded individuals with increasing wave exposure

TABLE II

Evidence for independent assortment of outer shell color and outer shell banding in Thais emarginata[1]

				Offspring phenotype[2]					
	Parent			'Orange'		Black			
Clutch label	Sex	Phenotype[2]	Inferred genotype[3]	Band	Unb	Band	Unb	χ^2	P
80-17-1A	m	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}	11	17	14	12	1.04	0.79
	f	Black, unband	OC^{BL}/OC^{BL}, OB^{U}/OB^{U}						
81-77-1A	m	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}	19	12	9	23	6.52	0.09
	f	Black, unband	OC^{BL}/OC^{BL}, OB^{U}/OB^{U}						
80-17-2-A1A	m	Black, banded	OC^{BL}/OC^{BL}, OB^{B}/OB^{U}	16	16	20	21	0.70	0.87
	f	Orange, unband	OC^{OR}/OC^{BL}, OB^{U}/OB^{U}						
80-17-2-A2A, B	m	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}	28	18	30	27	2.73	0.44
	f	Black, unband	OC^{BL}/OC^{BL}, OB^{U}/OB^{U}						
80-17-2-A6A	m	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}	39	17	23	7	4.39	0.22
	f	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}						
80-17-2-A12A	m	Black, banded	OC^{BL}/OC^{BL}, OB^{B}/OB^{U}	10	11	4	15	5.08	0.17
	f	Orange, unband	OC^{OR}/OC^{BL}, OB^{U}/OB^{U}						
80-17-2-A13A	m	Black, banded	OC^{BL}/OC^{BL}, OB^{B}/OB^{U}	24	24	16	17	2.11	0.55
	f	Orange, unband	OC^{OR}/OC^{BL}, OB^{U}/OB^{U}						
80-17-2-A15A	m	Black, unband	OC^{BL}/OC^{BL}, OB^{U}/OB^{U}	10	8	16	17	3.51	0.32
	f	Orange, banded	OC^{OR}/OC^{BL}, OB^{B},OB^{U}						
80-17-2-A16A	m	Black, banded	OC^{BL}/OC^{BL}, OB^{B},OB^{U}	3	3	3	6	0.87	0.83
	f	Orange, unband	OC^{OR}/OC^{BL}, OB^{U}/OB^{U}						
Group I[4]	—	Orange, banded	OC^{OR}/OC^{BL}, OB^{B}/OB^{U}	68	55	69	79	3.93	0.27
	—	Black, unband	OC^{BL}/OC^{BL}, OB^{U}/OB^{U}						
Group II[5]	—	Orange, unband	OC^{OR}/OC^{BL}, OB^{U}/OB^{U}	53	54	43	59	2.24	0.52
	—	Black, banded	OC^{BL}/OC^{BL}, OB^{B}/OB^{U}						
Pooled[6]	—	—	—	121	109	112	138	3.94	0.27

[1] Data only for crosses in which one or both parents were banded (OB^{B}/OB^{U}) and orange (OC^{OR}/OC^{BL}).

[2] 'Black' is a fairly discrete phenotype; 'Orange' individuals range from bright yellow-orange to orange-brown or dull orange with black mottling. 'Orange' individuals have been pooled because evidence from these and other crosses (Palmer, unpub.) strongly suggest this is a single allele whose phenotypic expression is influenced epistatically.

[3] See text for allele designations.

[4] 80-17-1A, 81-77-1A, 80-17-2-A2A, B, and -A15A pooled.

[5] 80-17-2-A1A, -A12A, -A13A, and -A16A, pooled.

[6] Group I and Group II, pooled.

(Table IV; Fig. 3). Rather curiously, banding frequency was not correlated with microhabitat type; at both Wizard Island and Cape Beale, the banding frequencies were not statistically different between samples taken predominantly or exclusively from within mussel beds (*Mytilus californianus*) or from among barnacles (Table V). At Cape Beale, unbanded individuals were statistically less common in 1984 than in 1983, but different areas were sampled in the two years, and the differences were small compared to those observed over the wave exposure gradient.

TABLE III

Color and banding phenotypes in F1 progeny from cross 82-52-1A (see also Fig. 2)[1]

Pigmentation		Number of offspring			Inferred genotype[2]		
Outer shell	Columella[3]	Strong banding	Weak banding	Un-banded	Shell color	Banding	Pigment reduction
Intense orange	or-br	9	—	—	OC^{OR}/OC^{BL}	OB^B/OB^B	OI^-/OI^-
Intense black	purp	10	—	1[4]	OC^{BL}/OC^{BL}	OB^B/OB^B	OI^-/OI^-
Pale orange	or-br	—	13	—	OC^{OR}/OC^{BL}	OB^B/OB^B	OI^-/OI^R
Pale brown	purp	—	12	—	OC^{BL}/OC^{BL}	OB^B/OB^B	OI^-/OI^R
Mostly unpigmented[5]	white	—	—	18	OC^{WH}/OC^{OR}	OB^B/OB^B	OI^-/OI^-, OI^-/OI^R
Mostly unpigmented[5]	purp	—	—	21	OC^{WH}/OC^{BL}	OB^B/OB^B	OI^-/OI^-, OI^-/OI^R

[1] Male = light grey with tan mottling and white lip, hint of banding (inferred genotype: OC^{WH}/OC^{BL}, OB^B/OB^B, OI^-/OI^R) Female = yellow-orange, banded (inferred genotype: OC^{OR}/OC^{BL}, OB^B/OB^B, OI^-/OI^-); see text for allele designations and alternative inferred genotypes.

[2] See text for allele designations.

[3] Abbreviations: purp = purple, or-br = orange-brown.

[4] Most likely an accidental contaminant (see text).

[5] Most individuals unpigmented, some with very pale yellow mottling.

DISCUSSION

The results presented above are all consistent with the hypothesis that spiral shell banding in *Thais emarginata* is controlled predominantly by a single autosomal locus (OB for outer shell banding) with two alleles, banded (OB^B) and unbanded (OB^U), and with the banded allele dominant (Table I). That banding should be controlled by a single locus is not surprising given what is known for the genetic control of shell banding in pulmonates (reviewed by Murray, 1975); in most pulmonates, the presence or absence of banding is controlled by a single locus, although the lack of bands may be either dominant [some helicids (2 spp.), Achatinidae (2 spp.)] or recessive [some helicids (2 spp.), Partulidae (1 sp.), Arionidae (1 sp.), Fructicicolidae (1 sp.)] depending on the species.

In contrast to pulmonates, however, the locus for shell banding appears to be unlinked to shell color (Table II; Palmer, 1984a) in *Thais emarginata.* Thus, although the occurrence of blocks of tightly linked genes for shell traits ('super genes') appears to be the rule among pulmonates (Murray, 1975; Murray and Clarke, 1976a, b), such tight linkage may not be as common among prosobranchs. Obviously, the generality of this conclusion must await additional genetic studies of prosobranch shell variation.

An additional difference between the results reported for pulmonates and those reported here is that, while the banding allele in pulmonates (B^B for *Cepaea nemoralis, C. hortensis,* and *Arianta arbustorum*) causes bands of a different pigment to be superimposed upon various ground colors of the shell (Murray, 1975), the banded allele in *Thais emarginata* (OB^B) causes a regularly spaced suppression of outer shell pigment. In *T. emarginata* from Barkley Sound, then, these 'bands' where pigment has been suppressed appear nearly white.

The results of cross 82-52-1A (Table III) were particularly interesting on two accounts. First, except for some variation within the two predominantly unpigmented forms, phenotypes were very discrete (Fig. 2a, b), suggesting that few loci were involved.

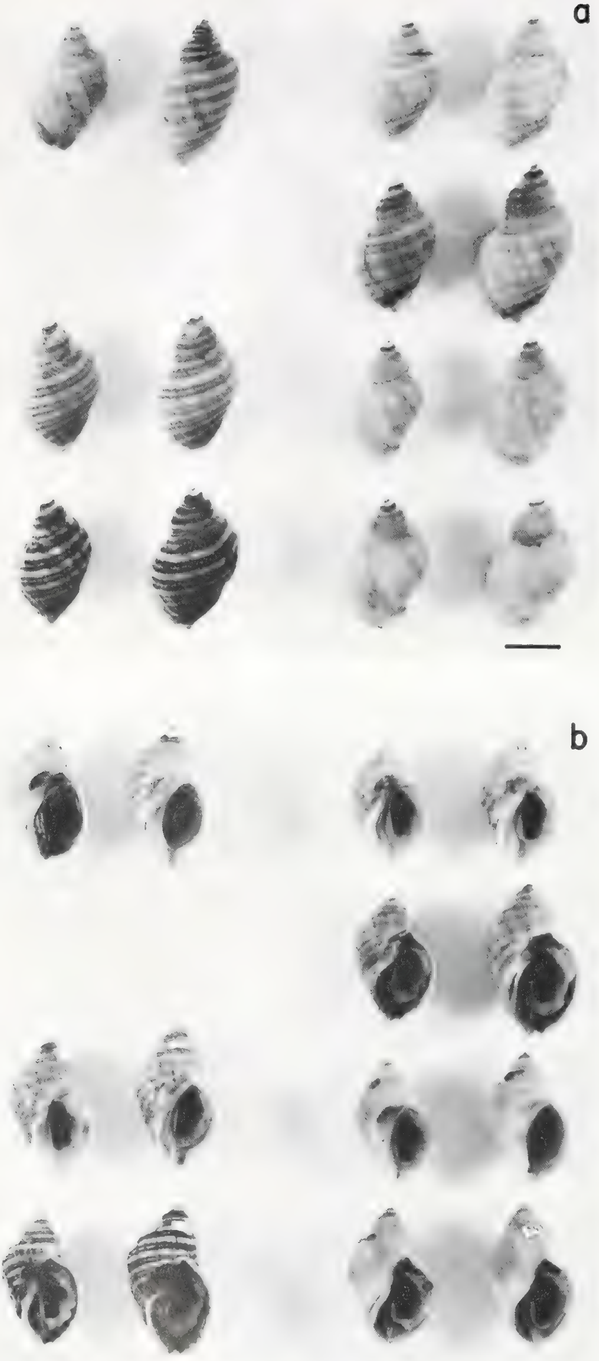

FIGURE 2. Possible evidence for pleiotropy; pigmentation and banding phenotypes from clutch 82-52-1A in *Thais emarginata.* (a) abapertural view, (b) apertural view of the same individuals. Columns 1 and 2: row 1—male parent = light grey shell with tan mottling and white lip, hint of banding, purple columella, female parent = yellow-orange shell and lip, banded, orange columella; row 3—both individuals intense

TABLE IV

Proportions of banding phenotypes in field populations of Thais emarginata *as a function of wave exposure, microhabitat type, and year*

Site	Date[1]	Wave exposure[2]	Micro-habitat[3]	Banded	Weakly banded	Unbanded[4]	N
Wizard Is. 1	9/83	38.8	Mostly M	0.09	0.18	0.73	147
Wizard Is. 2	9/83	38.8	Mixed M & B	0.08	0.13	0.79	132
Prasiola Pt.	6/84	63.4	B only	0.30	0.21	0.49	138
Cree Is.	6/84	75.1	B only	0.66	0.14	0.20	163
Cape Beale 1	7/83	80.3	Mostly B	0.62	0.08	0.31	131
Cape Beale 2	7/83	80.3	Mostly B	0.59	0.13	0.28	143
Cape Beale 3	7/83	80.3	Mixed M & B	0.63	0.13	0.24	134
Cape Beale 4	6/84	80.3	M only	0.61	0.22	0.17	87
Cape Beale 5	6/84	80.3	B only	0.61	0.22	0.17	128

[1] Month/year.

[2] Wave exposure index = entries are from the nearest station measured by Craik (1980): for Cree Is.—Benson Is., for Praisola Pt.—First Beach, for Wizard Is.—Helby Is.

[3] M—bed of *Mytilus californianus*, B—rock covered predominantly with *Balanus glandula*.

[4] Individuals with unpigmented shells pooled with pigmented, unbanded individuals.

Second, the offspring from this cross suggest very strongly that other alleles or loci influence the intensity of banding. The male parent was very pale grey with pale tan mottling and a white lip (= unpigmented) and at best only a hint of banding, and the female parent had a bright orange to yellow-orange, banded shell (leftmost two individuals respectively of row 1, Fig. 2a, b). One genetic hypothesis that accounts for the observed phenotype frequencies of the F1 assumes three, unlinked loci: (1) banding (male = OB^B/OB^B if female = OB^B/OB^U, or male = OB^B/OB^B, OB^B/OB^U or OB^U/OB^U if female = OB^B/OB^B), (2) outer shell color [male = OC^{WH}/OC^{BL}, female = OC^{OR}/OC^{BL} (OC^{WH} = 'white' or unpigmented, OC^{OR} = orange, OC^{BL} = black; in Vancouver Island populations $OC^{OR} > OC^{BL}$ in general, and at least one 'white' allele appears to exist with $OC^{WH} > OC^{OR} > OC^{BL}$ (Palmer, unpub.)], and (3) outer shell pigment intensity [one parent heterozygous OI^-/OI^R, one homozygous recessive (OI^R = partial reduction in pigment intensity in heterozygotes, complete reduction in homozygotes, OI^- = no reduction in pigment intensity; dominance is not known at present, so genotypes cannot be assigned to the parents)]. According to this model, the differences in columella color in the predominantly unpigmented individuals (two rightmost individuals of rows 3 and 4, Fig. 2a, b) would be determined by the allele carried in heterozygous condition with the proposed dominant white allele (OC^{WH}/OC^{OR} results in a white columella, OC^{WH}/OC^{BL} results in a purple columella). However, alternative interpretations of columella color variation are possible.

The above hypothesis derives from three aspects of the F1 phenotype frequencies. First, approximately half the offspring exhibited little or no outer shell pigmentation (bottom two rows, Table III), presumably because the dominant OC^{WH} allele from

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orange banded shell and lip, orange-brown columella; row 4—both individuals intense black banded shell and lip, purple columella. Columns 3 and 4: row 1—both individuals very faint orange banded shell and lip, orange-brown columella; row 2—both individuals very faint brown banded shell and lip, purple columella; row 3—both individuals unpigmented, white columella; row 4—both individuals unpigmented, purple columella. Only the largest two individuals from each phenotype were photographed. Actual phenotype frequencies in Table III. Scale bar = 10 mm.

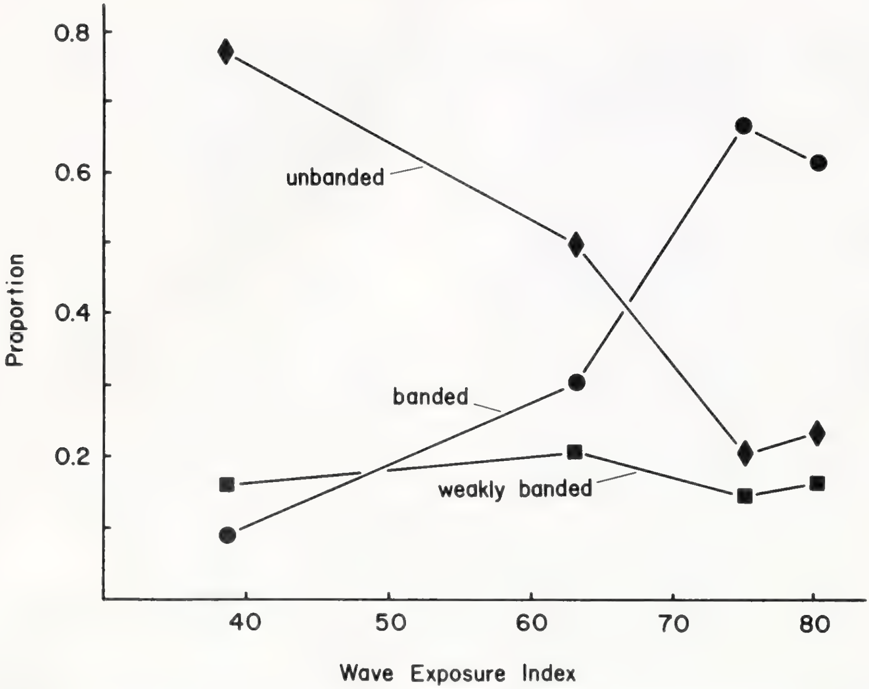

FIGURE 3. Change in proportion of banding phenotypes over a wave exposure gradient (data from Table IV).

the male suppressed expression of the OC^{BL} or OC^{OR} allele from the female parent, and the recessive OC^{BL} allele from the male allowed both female alleles to be expressed. Second, of the half of the clutch exhibiting distinct pigmentation, approximately half were strongly pigmented (upper two rows Table III) while the remaining half (rows 3 and 4) exhibited a similar color, but of much weaker intensity, presumably because half were homozygous recessive and half heterozygous at the pigment intensity locus (OI; until the dominance relationship is established at this locus, genotypes cannot be assigned). It is also possible that the presence of the mottled, pale yellow pigmentation in some of the 'unpigmented' individuals (rows 5 and 6, Table III) resulted from one of the alleles at this proposed pigment intensity locus. Third, all those offspring exhibiting distinct pigmentation showed some evidence of banding (upper four rows);

TABLE V

Results of contingency table analyses of banding frequency variation in Thais emarginata *(data in Table IV)*[1]

Comparison	χ^2	df	P	Sig
Between microhabitats, Wizard Is.	1.32	2	0.52	
Among microhabitats, Cape Beale, 1983	3.64	4	0.46	
Between microhabitats, Cape Beale, 1984	<0.01	2	>0.99	
Between years, Cape Beale	16.8	2	<0.001	***
Among sites	295.6	6	<0.001	***

[1] χ^2 = Chi-square value, df = degrees of freedom, P = exact probability, Sig = significance.

since outer shell pigmentation assorts independently from the banding locus (Table II; see also Palmer, 1984a), at least one parent had to have been homozygous dominant for banding. This last observation is significant, since it implies that all the weak or unpigmented individuals (bottom two rows) were also either heterozygous (OB^B/OB^U) or homozygous dominant (OB^B/OB^B) for banding even though they were phenotypically unbanded. Thus, banding may not be expressed in individuals carrying the 'unpigmented' allele for outer shell color (OI^R). If this interpretation is correct, these data contrast with those for *Cepaea nemoralis* (Cain *et al.*, 1968, cited in Murray, 1975) and *C. hortensis* (Cook and Murray, 1966) where individuals which are genetically 'banded' (*i.e.*, homozygous recessive) but do not produce any banding pigment (*i.e.*, 'transparent bands' = hyalozonate) nonetheless exhibit at least microstructural evidence of banding. No such 'transparent' bands appeared to be present in unpigmented, but presumably genetically banded individuals of *Thais emarginata.*

I should emphasize that alternative Mendelian models are also consistent with the phenotype frequencies in clutch 82-52-1A. Similar frequencies would obtain if (a) the proposed OC^{WH} allele was not dominant, but resulted in a dimunition of pigment intensity in heterozygotes (*e.g.*, rightmost two individuals of rows 1 and 2, Fig. 2a, b), and (b) the proposed pigment reducing allele (OI^R) was completely dominant to the normal allele (OI^-) instead of exhibiting partial reduction in pigment intensity in heterozygotes. This hypothesis requires that the parental genotypes be: male = OC^{BL}/OC^{WH}, OB^B/OB^B, OI^-/OI^R, female = OC^{OR}/OC^{BL}, OB^B/OB^B, OI^-/OI^-. Thus, with the present data, it is not possible to establish whether the predominantly unpigmented phenotype results (a) from a 'white' (= unpigmented) allele at the outer shell color locus (OC^{WH}), (b) from an independent allele at the proposed pigment reduction locus (OI^R), or (c) possibly from an allele at the banding locus (OB^S) that suppresses outer shell pigment uniformly about the mantle margin rather than in a regularly spaced manner as OB^B appears to do. Additional crosses are required to distinguish among these alternative hypotheses.

The evolutionary significance of the shell banding polymorphism in *Thais emarginata* is, unfortunately, uncertain at present. Although I have no direct evidence, the pronounced correlation of banding frequency with a gradient in wave exposure (Fig. 3), and the similarity of frequencies between samples taken one year apart at the same site compared to the frequencies at different sites (Tables IV and V), suggests rather strongly that the banding polymorphism is maintained by some form of natural selection. Presumably, selection is exerted by one or more species of visual predators, since visual predators are important sources of differential mortality in both marine gastropods [fish (Vermeij, 1978; Palmer, 1979; Reimchen, 1979; Mercurio *et al.*, 1985) and birds (Mercurio *et al.*, 1985, and references therein)] and terrestrial gastropods [birds (Cain and Sheppard, 1954; Jones, 1973)]. However, the lack of any correlation between banding frequency and microhabitat type at two sites (Tables IV, V) suggests such selection is not responsible for maintaining this polymorphism within a particular site. While several potential visual predators forage in habitats occupied by *Thais emarginata* in Barkley Sound [surfperch (*Damalichthys vacca, Embiotoca lateralis*), gulls (primarily *Larus californicus*), oystercatchers (*Haematopus bachmani*), turnstones (*Arenaria melanocephala*), and crows (*Corvus daurinus*); Mercurio *et al.*, 1985], the ones most responsible for maintaining the banding polymorphism in *T. emarginata* are unknown.

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EVIDENCE FOR A PROTON-ACTIVATED CHLORIDE CURRENT IN COELENTERATE NEURONS

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ABSTRACT

Neurons of the motor nerve net of *Cyanea* undergo a conductance change and depolarize when stimulated with saline at pH 5.5 or lower. The typical response to a 10–20 ms pulse of acidic saline is a brief depolarization whose amplitude is dependent on the resting potential. The reversal potential is −25 mV. Alterations in the extracellular concentrations of Na^+ and K^+, and variations in the pH of the stimulating saline had no effect on the reversal potential. Alterations in the extracellular Cl^- concentration alone affected the reversal potential suggesting that the response is a proton-activated chloride efflux. This sensitivity to protons was uniform over the entire cell.

INTRODUCTION

The motor nerve net of the jellyfish *Cyanea capillata* is a two dimensional plexus of bipolar neurons that transmits swimming activity from marginal pacemaker centers, the rhopalia, to the swimming muscle (Anderson and Schwab, 1981, 1983, 1984). Neurons within the nerve net are connected by morphologically symmetrical chemical synapses (Anderson and Schwab, 1981); the bidirectionality implied by this organization has recently been confirmed physiologically (Anderson, 1985). While a great deal is now known about the physiology of neurons in this and other coelenterate species (for reviews see Anderson and Schwab, 1982; Passano, 1982; Shelton, 1982; Spencer and Schwab, 1982), one major question that remains generally unresolved is the identity of the neurotransmitter present at chemical synapses in these organisms (for review see Martin and Spencer, 1983).

During experiments designed to identify the neurotransmitter at synapses between motor nerve net neurons in *Cyanea,* we noted that acidic substances consistently depolarized the neurons. These responses were transient and closely resembled those that might have been expected upon application of the neurotransmitter. As will be seen, however, the reversal potential of these depolarizations was inconsistent with that of the postsynaptic potentials (EPSPs) at these synapses, +4 mV (Anderson, 1985). Nevertheless, the similarity between the waveform of the evoked response and that which would be expected as a result of neurotransmitter action prompted this study of the properties of these pH evoked depolarizations.

MATERIALS AND METHODS

Neurons of the motor nerve net of the scyphomedusan jellyfish *Cyanea capillata* were exposed by brief oxidation of the overlying myoepithelium (Anderson and Schwab, 1984). The exposed neurons were then bathed in *Cyanea* saline (see below) and maintained at 9°C. These preparations were used within 2–3 days post-exposure.

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Intracellular recordings were obtained using patch pipettes in the whole-cell, current-clamp configuration of the patch clamp technique (Hamill *et al.*, 1981). Details of the procedures, as applied to the motor nerve net neurons of *Cyanea,* are presented elsewhere (Anderson, 1985).

Substances to be tested were loaded into patch pipettes similar to those used for recording and ejected using 10–20 ms pulses of nitrogen at 20–40 p.s.i. under the control of a Picospritzer (General Valve Corporation). These parameters resulted in ejection of 60–70 pL of solution. The tip of the pipette was positioned 20–40 μm from the cell and while every attempt was made to ensure that the pipette was aimed at the cell, it was sometimes difficult to gauge the vertical position of the pipette tip relative to the cell. Discrepancies here resulted in abnormally small responses. These could usually be rectified by repositioning the pipette.

Records of evoked responses were recorded on video tape (Bezanilla, 1985) and analyzed using a Nicolet 2900 Digital oscilloscope.

Patch pipettes were filled with a low Ca^{++}, high K$^+$ solution of the following composition (m*M*): KCl 140; EGTA 11; HEPES 10; CaCl$_2$ 1; glucose 696. The solution was adjusted to pH 7.0 with KOH, giving a final [K$^+$] of 210 m*M*.

The composition of the various extracellular media used are given in Table I. Unless otherwise stated, all external solutions were adjusted to pH 7.4. All experiments were conducted at room temperature.

RESULTS

Application of a 10–20 ms pulse of *Cyanea* saline at pH 5.4 to exposed neurons of the motor nerve net produced a brief depolarization of the cell (Fig. 1A). The amplitude of the response was somewhat variable depending in part on the accuracy with which the sample was directed at the cell. At resting potentials of −60 mV, the normal resting potential of these cells (Anderson and Schwab, 1983), depolarizations of 30 to 40 mV were common. Repetitive applications produced similar responses (Fig. 1B); their amplitude decreased when pulses were applied at frequencies in excess of 1 Hz but generally, at 1 Hz, the amplitude was relatively constant. The apparent

TABLE I

*Composition of solutions (m*M*)*

		Solution			
Salt	*Cyanea* saline	50% Na	10% K	122 m*M* Cl	41.5 m*M* Cl
NaCl	390	195	403	—	—
KCl	13.4	13.4	1.34	13.4	—
CaCl$_2$	9.5	9.5	9.5	9.5	—
MgCl$_2$	24	24	24	24	—
MgSO$_4$	5	5	5	5	5
Choline Cl	41.5	41.5	41.5	41.5	41.5
HEPES	10	10	10	10	10
TMACl	—	195	—	—	—
Na asparate	—	—	—	390	390
K gluconate	—	—	—	—	13.4
Ca gluconate	—	—	—	—	9.5
Mg gluconate	—	—	—	—	24

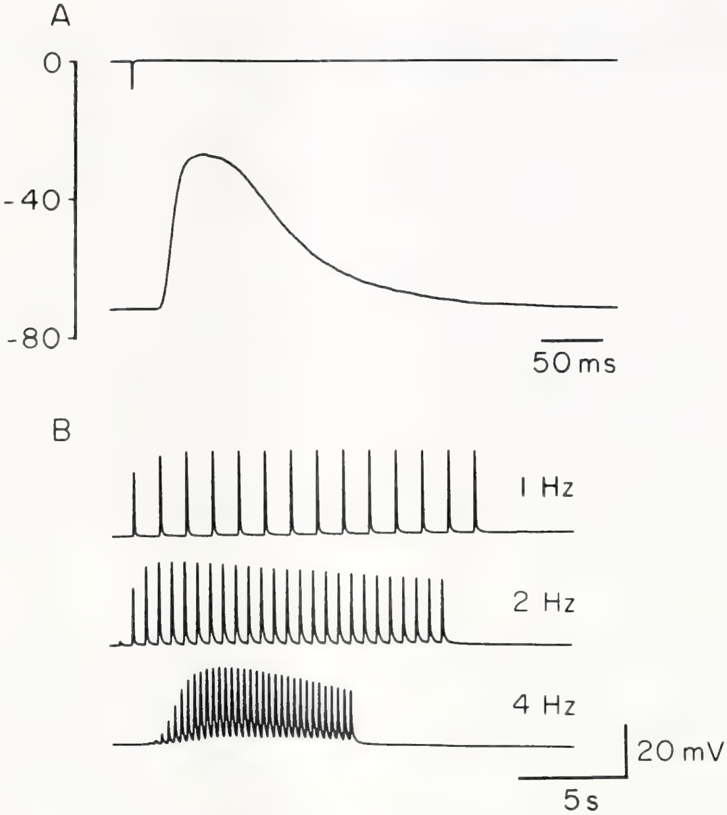

FIGURE 1. (A) Depolarization of a motor nerve net neuron produced by a brief pulse of acidic saline. The downward deflection on the zero millivolt line (upper trace) indicates the onset of the stimulus pulse. (B) The effect of repetitive applications to the same neuron at three frequencies. The gradual increase in response amplitude was not observed consistently; the decrease at high frequency was typical.

facilitation at the onset of these series are atypical and probably reflect dilution of the pipette contents prior to the onset of the stimulus trains.

These depolarizations occurred irrespective of the type of acid as long as the pH was 5.4 or less. Both organic (acetic, formic, HEPES, MES) and inorganic (HCl, HNO_3) acids produced comparable responses. Saline at normal pH (7.4) produced no obvious effect other than occasional small mechanical artefacts.

The typical response evoked by a single 10 ms application of saline (pH 5.4) had a fast rising phase and a slower repolarization. For the depolarization shown in Figure 1A the rising phase had a maximum slope of 6.4 V/s. Peak amplitude was reached 17.8 ms after the onset and repolarization took 187 ms. The rate of rise and peak amplitude of the responses varied according to the position of the acid-containing pipette; the greater the separation between the recording and stimulus pipettes, the smaller and slower the response. Accordingly, useful quantitative data on the waveform of individual responses is difficult to provide. The depolarization presented in Figure 1A is typical of those evoked by a pipette positioned close to the recording site, the soma. The entire response typically lasted for slightly longer than the duration of the applied pulse (whatever the position of the stimulus pipette). With longer duration stimuli, the response peaked and then decreased in amplitude during the stimulus. The latency of the response, measured from the onset of the electrical stimulus used to drive the Picospritzer (upper trace, Fig. 1A) was from 12–20 ms irrespective of electrode placement. This delay is obviously an over-estimate of the true latency since part of that time is required for ejection of the stimulus.

The absolute amplitude and polarity of the pH evoked response was strongly dependent on the resting potential of the cell (Fig. 2A). The membrane potential was changed by injecting current into the cell through one arm of a Wheatstone bridge. The bridge balance was continuously monitored to ensure that the displayed resting potentials were accurate. Pulses of acidic saline were then applied to the cell over a range of resting potentials. The position of the stimulus pipette remained constant during the series. The relationship between resting potential and response amplitude (Fig. 2B) was linear with slopes typically slightly less than unity (Mean = 0.77). The reversal potential was typically in the range −20 to −30 mV (Mean = −25.43 ± 1.71 S.E.M.).

Injection of hyperpolarizing pulses into the cell during application of the stimuli revealed that the pH evoked depolarizations were associated with a conductance increase (Fig. 2C). This conductance increase is in excess of that attributable to the delayed rectifier known to be present in these cells (Anderson and Schwab, 1984) as evidenced by the fact that when hyperpolarizing pulses were applied over a range of resting potentials the reductions in hyperpolarizing pulse amplitude were far less than those that occurred during application of acidic saline (Fig. 2C).

Ionic basis of the response

When cells were bathed in normal *Cyanea* saline (pH 7.4), application of *Cyanea* saline (pH 5.4) evoked a depolarization whose mean reversal potential was −25.43

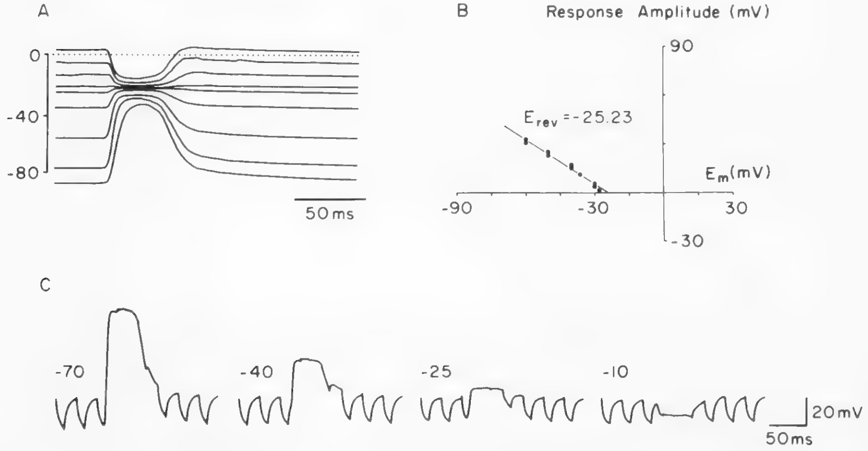

FIGURE 2. (A) A series of pH evoked depolarizations recorded from the same cell at a variety of resting potentials. The site of stimulation and stimulus parameters were the same throughout the series. Note that the polarity of the response changes at around −20 mV. (B) A similar series from another cell plotted to show the reversal potential. (C) A series of pH evoked depolarizations recorded as in (A) with the addition of superimposed hyperpolarizing current pulses. Note that while the amplitude of the hyperpolarizing voltage responses decrease slightly at less negative resting potentials, the decrease in amplitude is far less than that accompanying the pH-induced depolarization. The resting potential for each record is given at the left side of each record.

mV. The ionic basis of the response was examined by assessing the effect of ionic substitutions on that reversal potential (Fig. 3A). In 50% Na saline the average reversal potential of the response evoked by saline (pH 5.4) was −24.13 (±3.29 S.E.M.) mV. Similarly, application of 50% Na saline (pH 5.4) to neurons bathed in saline (pH 7.4) produced responses which reversed at −20.28 (±2.58 S.E.M.) mV. In the case of K^+, cells were bathed in saline that contained $^1/_{10}$th normal K^+ (1.34 m*M*). Responses evoked by saline at pH 5.4 reversed at −20.1 (±1.23 S.E.M.) mV while those evoked by application of low K^+ saline (pH 5.4) to neurons bathed in *Cyanea* saline (pH 7.4) produced responses which reversed at −23.85 mV. These values for reversal potential are all within the normal range of reversal potentials for the normal response and, therefore, suggests that neither Na^+ nor K^+ is the charge carrier for the depolarization. Similarly for H^+. Application of saline at pH 4.4 evoked similar depolarizations with reversal potentials of −18.2 (±0.05 S.E.M.). While slightly lower than those typical of the normal responses the difference is insignificant compared to the 58 mV change one would expect for a 10-fold change in $[H^+]_0$.

Reductions in $[Cl^-]_0$ did, however, produce major changes. The depolarization evoked by application of acidic saline were invariably larger and the reversal potentials were altered. When cells bathed in normal saline (pH 7.4) were stimulated with salines in which $^1/_2$ and $^1/_{10}$ of the normal Cl^- was replaced with asparate, evoked responses had reversal potentials of −13.3 (±0.6 S.E.M.) mV and −3.4 mV, respectively. Since it is difficult to exclude mixing between the applied saline and that present in the bath, the absolute $[Cl^-]_0$ concentration in these trials cannot be ascertained. The dependency of the reversal potential on the $[Cl^-]_0$ was confirmed by repeating these experiments with cells bathed in 41.5 m*M* Cl^- saline (pH 7.4) and applying pulses of the same low-Cl^- saline at pH 5.4. Under these conditions, the average reversal potential was +28.47 (±1.62 S.E.M.) mV. This translates into a 43.7 mV change in reversal potential for a 10-fold change in $[Cl^-]_0$. This is smaller than that predicted by the Nernst equations (58 mV/10-fold change) but the magnitude of the change suggests that the pH induced depolarization is due, at least in part, to movement of Cl^-.

In low $[Cl^-]_0$, applications of acidic saline produced large responses which overshot zero and usually evoked an action potential (Fig. 3B). Interestingly, spike threshold was more negative in low-Cl^- than in normal saline (−25 mV as opposed to approximately OmV). Repetitive applications of low pH saline to neurons bathed in 41.8 m*M* $[Cl^-]_0$ produced a train of depolarizations most of which gave rise to an action potential. The depolarizations decreased in amplitude as before but at a slightly faster rate.

Spacial distribution of sensitivity

To evaluate the spacial distribution of this response, acidic saline was applied at discrete points along the length of one axon of a cell. Depolarizations occurred when any part of the cell was stimulated, but, as indicated earlier, the amplitude of the evoked response and the rate of depolarization decreased with distance from the recording site. A plot of $\log_e$ response amplitude against distance (Fig. 4) for the responses evoked at different positions on a cell bathed in 41.8 m*M* Cl^- (pH 7.4) saline and stimulated with the same saline (pH 5.4) was linear (R = 0.987) with a slope of −5.39.

Blocking agents

When experiments of the type described above were repeated on cells bathed in 41.8 m*M* Cl^- saline that contained 2 m*M* 4-Acetamido-4′-isothiocyanostilbene -2,2′-

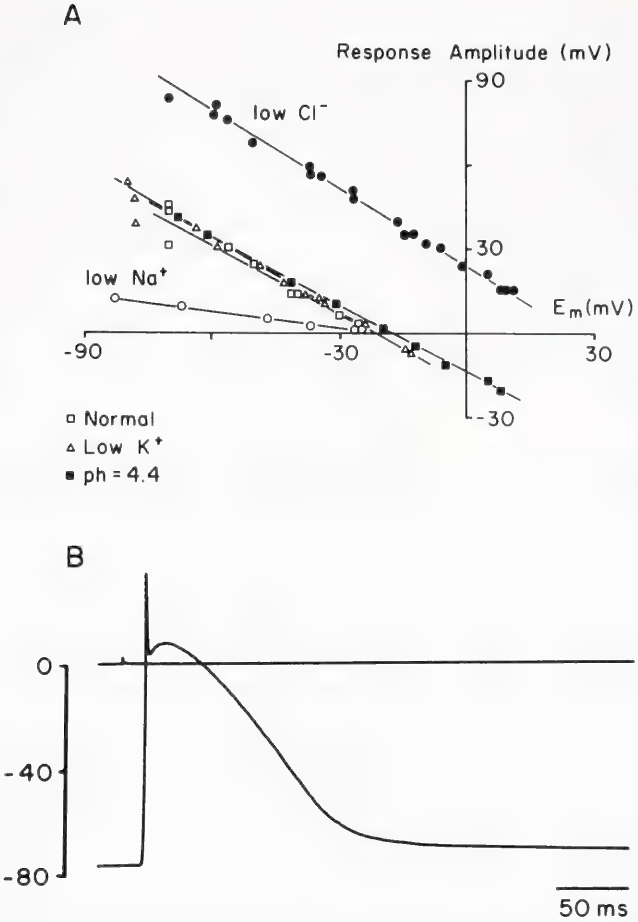

FIGURE 3. (A) The effect of changes in the ionic composition of the saline on the reversal potential of the pH-induced depolarization. Each series is from a different cell. Note that changes in extracellular $[Cl]_0$ alone affect the reversal potential. Variations in the slope of the lines merely reflect different stimulus positions. (B) A single pH-evoked depolarization recorded in low Cl saline. In this medium the depolarization evoked a significantly larger depolarization which typically produced a single action potential. The stimulus marker is given on the zero millivolt line (upper trace).

Disulfonic acid (SITS) responses were reversibly abolished. The effect was use dependent; the first of a train of stimuli usually evoked small ($\leq$5 mV) depolarizations but subsequent stimuli produced progressively smaller responses. However, the nerve net invariable deteriorated very rapidly when bathed in SITS-containing saline. The neurons detached from the mesoglea and the axons appeared to become finer and retract. These structural changes put the usefulness of SITS as a blocking agent in doubt. Extracellular Cd^{++} (5 mM) was ineffective.

DISCUSSION

This study was designed to evaluate the mechanisms responsible for the depolarization produced by application of acidic saline to neurons from the jellyfish *Cyanea*.

It was undertaken partly because of the similarity between the waveforms of the pH response and those that might be expected upon application of the correct neurotransmitter and partly because the presence of this response precludes the application of neurotransmitter candidates at acidic pH. This latter capability may be particularly important. To date we have applied a total of 25 recognized neurotransmitter candidates to synapses between these neurons (Anderson and McKay, unpub.). All have been applied at pH 7.4 to avoid activation of the pH response and in no case has any response indicative of neurotransmitter action been observed. However, the contents of most, if not all, synaptic vesicles is acidic (Russell and Holtz, 1981; Russell, 1984; Gainer *et al.*, 1985) and it is conceivable that the neurotransmitters at these synapses is active only at acidic pH. It is possible that at pH 7.4 the transmitter may be relatively inactive, in which case its application at synapses would not produce a noticeable response. However, if the pH-evoked depolarization could be blocked then candidate transmitters could be applied at acidic pH. This study was undertaken with this end in mind.

It is clear from the data presented here that the reversal potential of the pH-evoked response is insensitive to changes in $[Na^+]_0$ and $[K^+]_0$ and, so long as the pH is 5.5 or less, insensitive to the pH of the applied substance. Only when $[Cl^-]_0$ was altered was the reversal potential of the response affected implying that the response is due to a Cl^- current, specifically a Cl^--efflux since the response is one of depolarization. The effect of changing $[Cl^-]_0$ was to alter the reversal potential by 44 mV/10-fold change in $[Cl^-]_0$. This is less than the 58 mV predicted by the Nernst equation. The discrepancy may be due to the movement of other ions not examined here or, alternatively, could be explained by incomplete solution mixing or significant local changes in $[Cl^-]$ during the response.

Acidic saline applied to any part of the cell produced a typical depolarization. This suggests that the pH sensitivity is a widespread phenomenon and not localized. However, the amplitude of the response decreased as the separation between the recording and stimulating electrode increased. If the distribution of sensitivity is uniform on the entire cell, the rate of decrease should be a measure of the space constant of these cells. Axons of the motor nerve net are prohibitively small for multiple axonal penetrations of the type necessary for measurements of space constant but, based on the dimensions of the cells and the known membrane constants of excitable cells from other coelenterates, the space constant has been conservatively estimated to be 600 μm (Anderson and Schwab, 1983). The apparent space constant (190 μm) derived from the inverse slope of the decay of the pH-evoked depolarization (Fig. 4), is less than that previously estimated but appropriate to bipolar neurons with axon diameters in the range 1–5 μm. Since these experiments involved the use of depolarizing signals which probably activate specific conductances, the value of the space constant is probably less than the resting space constant which would typically be obtained using hyperpolarizing pulses.

Proton-activated chloride channels of the type envisaged here have been reported on several occasions in muscle (Loo *et al.*,1980; Klein, 1985) and modified muscle, electroplax (Hanke and Miller, 1983). In *Torpedo* electroplax, such channels occur in high density on the non-innervated face of the muscle and their function remains unclear. In skeletal muscle, on the other hand, chloride channels are widespread and may serve to confer stability on the cells since, in their absence, the muscles become hyperexcitable (Bryant and Morales-Aquilera, 1971). Interestingly, Cl^- currents in skeletal muscle (Vaughan and Fong, 1978) and electroplax (Hanke and Miller, 1983) are sensitive to SITS, as are those described here.

Despite the apparent prevalence of chloride channels in muscle, however, there has been only one report of chloride channels in neurons (Franciolini *et al.*, 1985).

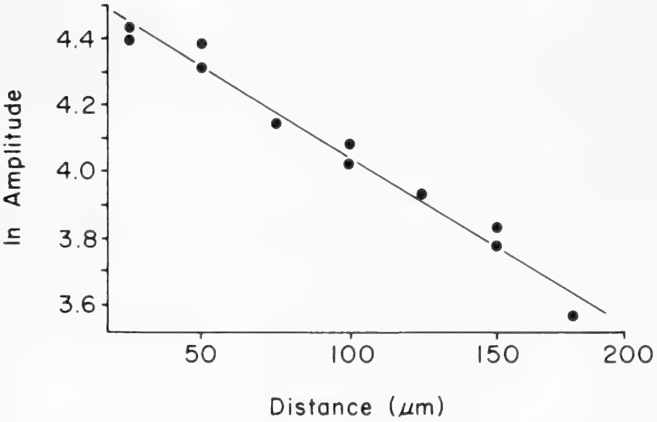

FIGURE 4. A semilog plot of the amplitudes of responses evoked at different distances along the length of an axon. The slope of this line translates into a space constant of 190 μm.

Single channel analysis of patches from cultured central neurons from the rat showed that the channels are active at voltages between −60 and +60 mV and pass outward currents more easily than inward currents. The pH sensitivities of those channels were not, however, examined. Proton-activated depolarizations similar to those described here have been reported in cultured mammalian neurons impaled with KAc-filled microelectrodes (Gruol *et al.*, 1980). The underlying mechanism was not studied in detail but it was noted that while the response was insensitive to tetrodotoxin (TTX) and Mn^{++}, suggesting that the effect was not produced by movements of Na^+ or Ca^{++} respectively, it became far more complex when KCl-filled electrodes were employed, suggesting a role of Cl^-. Unfortunately, the effect of changes in $[Cl^-]_0$ was not examined.

The role of the chloride current in *Cyanea* neurons is unclear. Interestingly, one effect of lowering the $[Cl^-]_0$ was to make spike threshold more negative thereby facilitating electrogenesis. This suggests that the chloride current in these cells might serve as a way of controlling the excitability of these neurons in the same way as has been suggested for skeletal muscle (Bryant and Morales-Aquilera, 1971). The role of pH in this process is, however, unknown. It has been shown that single chloride channels from electroplax (Hanke and Miller, 1983) are opened by external protons but the functional significance of this, if any, is unclear.

The similarity between the responses described here and those reported elsewhere are very marked suggesting a high degree of conservation of the phenomenon through evolution. However, the degree of conservation will become apparent only with examination of the individual chloride channels.

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THE EFFECTS OF β1,3-GLUCANS ON BLOOD COAGULATION AND AMEBOCYTE RELEASE IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

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ABSTRACT

The best-studied defense reaction in the horseshoe crab, *Limulus polyphemus,* involves the exocytotic release of a clotting system from the blood cells (amebocytes) and the formation of an extracellular clot in response to bacterial lipopolysaccharide. Although β1,3-glucans, apparently serving as signals for the presence of potentially pathogenic fungi, trigger release, blood coagulation, and activation of the prophenoloxidase system in many arthropods, *Limulus* appears to be unresponsive to endotoxin-free preparations of naturally occurring β1,3-glucans. However, the carboxymethylated β1,3-glucan derivative CMPS induced gelation of amebocyte lysates. The induction of clotting enzyme activity and gelation of amebocyte lysate by CMPS followed a complex pattern. Enzymatic activity generated by CMPS was only 15–42% of that produced by bacterial lipopolysaccharide. Neither native β1,3-glucans nor CMPS induced exocytosis of living *Limulus* amebocytes. We conclude that although CMPS produced coagulation of amebocyte lysates, the response is of questionable biological significance since native β1,3-glucans were inactive. The blood of *Limulus* lacked detectable phenoloxidase activity.

INTRODUCTION

Arthropods possess a variety of defense reactions to potentially pathogenic bacteria and fungi that gain access to the internal milieu through wounds or by active invasion through the integument (Salt, 1970; Lackie, 1980; Ratcliffe *et al.*, 1982). Many of the well studied systems involve activities of the blood cells (Bang, 1967; Levin, 1967, 1976; Ratcliffe *et al.*, 1982; Armstrong, 1985). These include phagocytosis and release of various antimicrobial systems by the exocytosis of cytoplasmic granules (Armstrong and Levin, 1979; Nachum, 1979; Nachum *et al.*, 1979; Armstrong and Rickles, 1982). The prophenoloxidase activating system appears to be an important representative of the latter category in many arthropods (Söderhäll, 1982; Söderhäll and Smith, 1984). In *Limulus,* exocytosis results in the release from the blood cells (amebocytes) of a clotting system consisting of a clottable protein (coagulogen) and a system of proteinases (Levin and Bang, 1968; Young *et al.*, 1972). The ultimate member of the proteinase cascade system (clotting enzyme) converts coagulogen, *via* a limited proteolytic cleavage, into a protein capable of polymerizing into a gel (Young *et al.*, 1972; Nakamura and Levin, 1982a, b; Torano *et al.*, 1984). This presumably functions in defense by sealing defects in the circulatory system and immobilizing microbes, thus preventing their systemic dissemination throughout the circulation (Levin and Bang, 1964a, b).

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Elements of the cell walls of pathogenic organisms appear to function as signals to host defense systems that microbes are present. Bacterial lipopolysaccharide (LPS, endotoxin) serves as an extracellular signal for the presence of gram-negative bacteria and β1,3-glucans serve as a signal for the presence of fungi. LPS is a potent activator of defense processes in *Limulus:* the blood cells can be stimulated to undergo exocytosis by LPS (Levin and Bang, 1964a; Armstrong and Rickles, 1982) and the system of proteinases involved in clotting of the coagulogen released from amebocytes also is activated by LPS (Levin and Bang, 1968; Young *et al.*, 1972; Levin, 1979). A variety of crustaceans demonstrate activation of phagocytosis, exocytosis, and the prophenoloxidase system both by LPS and by β1,3-glucans (Söderhäll and Unestam, 1979; Söderhäll, 1982, 1983; Smith and Söderhäll, 1983a, b; Söderhäll and Häll, 1984). Of interest to the present study is the possible role played by β1,3-glucans in defense reactions of blood cells of *Limulus polyphemus,* the horseshoe crab.

MATERIALS AND METHODS

Limulus *amebocyte lysate*

Amebocyte lysates from the horseshoe crab, *Limulus polyphemus,* were prepared using previously described methods (Levin and Bang, 1968; Young *et al.*, 1972).

Cancer *hemocyte lysate*

Hemocyte lysates from *Cancer borealis* were prepared by withdrawing approximately 5 ml of hemolymph from individual crabs into 10 ml syringes that contained 2.5 ml of ice-cold anticoagulant (0.45 *M* NaCl; 0.1 *M* glucose; 30 m*M* trisodium citrate; 26 m*M* citric acid; and 10 m*M* Na-EDTA; at pH 4.6) (Söderhäll and Smith, 1983). The sample was centrifuged at 800 × *g* for 10 min, and the pellet washed once with 10 ml of anticoagulant. The hemocyte pellet was then resuspended in 1 ml of 0.01 *M* sodium cacodylate buffer, pH 7.0, homogenized, and spun at 2000 × *g* for 20 min to remove cell debris. The resulting supernatant was used as a source of the prophenoloxidase activating system (Söderhäll, 1982).

Astacus *hemocyte lysate*

Hemocyte lysates from the crayfish, *Astacus astacus,* were made as described by Söderhäll (1983).

Glycans

The following glycans were obtained from commercial suppliers: laminarin, a crude β1,3-glucan (Calbiochem Co., La Jolla, California); zymosan (yeast cell walls, Sigma Chemical Co., St. Louis, Missouri); hyaluronic acid (Sigma); dextran T10, T20, T40, T70, and T500 (Pharmacia, Uppsala, Sweden); cellulose (Kebo, Stockholm, Sweden); starch (Merck, Darmstadt, West Germany); and mannitol and glucose (Merck). Chitin was prepared according to Sneath (1960). Laminarin pentaose, laminarin G, and laminarin M were purified and their chemical structure elucidated as reported by Söderhäll and Unestam (1979). All glycans were dissolved or suspended in pyrogen-free distilled water.

A carboxymethylated β1,3-glucan (CPMS) with a degree of polymerization of about 540 and a degree of substitution of 0.68 was a generous gift from Dr. A. Kakinuma, Takeda Chemical Industries, Ltd., Osaka, Japan, and Dr. S. Iwanaga, Kyushu University, Fukuoka, Japan.

Endotoxin assay

Endotoxin contamination of buffers or solutions was examined using the gelation reaction of *Limulus* amebocyte lysate, as previously described (Levin and Bang, 1968). Endotoxin (lipopolysaccharide B) from *E. coli* 026:B6 was used as a standard (Difco Laboratories, Detroit, Michigan).

Assay of clotting enzyme activity in Limulus *amebocyte lysate*

Clotting enzyme activity was assayed either by the time required to form a gel at 37°C (Levin and Bang, 1968) or by measuring the generation of hydrolyzing activity toward the chromogenic synthetic peptide, Ac-Ile-Glu-Gly-Arg-pNA HCl (S-2423, AB Kabi Vitrum, Stockholm, Sweden). Clotting enzyme hydrolyzing activity, induced by different potential activators, was measured by incubating 50 μl of amebocyte lysate, 50 μl of activator, 400 μl of 0.05 *M* Tris-HCl buffer in 0.45 *M* NaCl (pH 8.0), and 100 μl of 2 m*M* S-2423 at 37°C (usually for 30 min). To stop the reaction, 100 μl of 50% acetic acid was added. The absorbance at 405 nm was measured, and enzyme activity generated in the reaction mixture is expressed as absorbance per min of incubation (A_{405}/min) (Nakamura and Levin, 1982a).

Clearly it is important to remove or inactivate endotoxin from the β1,3-glucans when testing their ability to activate the *Limulus* clotting system, since the clotting reaction is so sensitive to endotoxin that low levels of contaminating endotoxin can cause clotting even when the reagent being investigated is itself inactive (Cutler *et al.*, 1972; Rickles *et al.*, 1977, 1979; Zuckerman *et al.*, 1979). In the present study, this was accomplished by the inclusion of polymyxin B, which binds and inactivates endotoxin (Cooperstock and Riegle, 1981; Duff and Atkins, 1982; Nakamura and Levin, 1982a), and by fractionation of laminarin into the M and G chains (Söderhäll and Unestam, 1979).

Preparation of crude clotting enzyme and coagulogen

A preparation of crude clotting enzyme was generated by incubating *Limulus* amebocyte lysate with *E. coli* endotoxin (final concentration, 1 μg/ml) for 4 h at 37°C, followed by 20 h incubation at 4°C. The solid gel that formed as the result of the reaction was removed by centrifugation at 27,500 × *g* for 20 min at 4°C in a Sorvall 2-B refrigerated centrifuge. The enzyme preparation has been demonstrated previously to lack coagulogen (Young *et al.*, 1972) and is capable of gelling coagulogen (the clottable protein) present in amebocyte lysate.

A preparation of coagulogen was obtained by heating *Limulus* amebocyte lysate at 65°C for 20 min in a water bath, as described previously (Young *et al.*, 1972). The flocculent material that was produced was removed by centrifugation for 15 min at approximately 2000 × *g*. The coagulogen preparation lacked detectable clotting enzyme activity since addition of endotoxin to the coagulogen failed to produce gelation. However, incubation of the preparations of clotting enzyme and coagulogen produced a solid gel. The reaction between these preparations was not endotoxin-dependent, because it was not blocked by polymyxin B (at concentrations as high as 66,000 U/ml), a known inhibitor of bacterial endotoxins (Duff and Atkins, 1982).

Assay of phenoloxidase activity

Phenoloxidase activity in *Cancer* hemocyte lysates or *Limulus* amebocyte lysates was assayed by preincubating 100 μl of lysate with 100 μl of activator (β1,3-glucans) for 10 min at 20°C. To this reaction mixture was then added 100 μl $MgCl_2$ (50 mM); 50 μl $CaCl_2$ (10 mM); 100 μl 0.1 M sodium cacodylate buffer (pH 7.0), and 100 μl L-dopa (1 g/l) (dihydroxyphenyl-alanine; Sigma Chemical Co., St. Louis, Missouri). These conditions are optimal for phenoloxidase activity in several marine crustaceans (Smith and Söderhäll, 1983a, b; Söderhäll and Smith, 1983). Appropriate controls always were concomitantly performed, in which pyrogen-free distilled water (Cutter Laboratories Inc., Berkeley, California) was substituted for the activator. The enzymatic reaction was allowed to proceed for different lengths of time at 20°C and enzyme activity is expressed as absorbance at 490 nm/minute.

Assay of protease activity in crayfish hemocyte lysate

The protease activity of hemocyte lysate supernatants (70,000 × *g*, 20 min) was assayed using the chromogenic peptide, Bz-Ile-Glu-(γ-O-piperidyl)-Gly-Arg-pNA (S-2337, AB Kabi Vitrum, Molndal, Sweden) as substrate, as described by Söderhäll (1983).

Miscellaneous

Other chemicals used were: polymyxin B (Burroughs Wellcome Co., Triangle Park, North Carolina) and L-dihydroxyphenyl-alanine (Sigma). All other chemicals were of analytical grade. All glassware was rendered sterile by autoclaving at 150°C for 45 min and then made pyrogen-free by heating in a dry oven at 190°C for 4 h.

Exocytosis

Exocytosis was studied by direct microscopic observation of amebocytes adherent to microscope coverglasses mounted in a simple perfusion chamber, as described by Armstrong and Rickles (1982). Five to ten drops of blood were diluted in 15 ml of sterile, endotoxin-free 3% NaCl, contained in an endotoxin-free 90 mm petri dish with 4–6 22 mm coverglasses on the bottom. The cells settle and attach to the coverglasses in 1–5 min, which can then be transferred to a perfusion chamber constructed by inverting a coverglass over a drop of saline on a sterile, endotoxin-free microscope slide. The coverglass is supported above the slide on fragments of a No. 1½ coverglass. The cells can then be presented with various agents dissolved in 3% NaCl that is perfused beneath the coverglass. The progress of degranulation was ascertained by periodic microscopic examination (Armstrong and Rickles, 1982).

RESULTS

Induction of clotting enzyme activity by natural glycans

Several glycans were tested for their ability to induce clotting enzyme activity in *Limulus* amebocyte lysate, using the gelation assay (see Materials and Methods) or by recording hydrolysis of the synthetic peptide, Ac-Ile-Glu-Gly-Arg-pNA (S-2423). Of the natural glycans tested, only a crude β1,3-glucan (laminarin) induced significant clotting enzyme activity in amebocyte lysate. Zymosan, a yeast cell wall preparation, only slightly affected the system, and all other natural glycans tested were negative (Table I). The optimal concentrations of laminarin for activation were greater than 0.01 mg/ml.

Interestingly, two purified subfractions of laminarin, laminarin G (Lam G) and laminarin M (Lam M) (Söderhäll and Unestam, 1979), were inactive. The former is

TABLE I

Effects of different glycans on clotting enzyme activity of Limulus *amebocyte lysate*

Glycan	Optimal concentration (mg/ml)	Structure	Amidase activity (ΔA_{405} nm/min/ml reaction mixture)
Laminarin	10^{-1}	(1–3) β-D-glucan	0.14
CMPS[1]	10^{-4}	(1–3) β-D-glucan, carboxymethylated	0.032
Zymosan	10^{-4}	yeast cell walls	0.006
Laminarin G	Not active[3]	(1–3) β-D-glucan terminated with glucose	0
Laminarin M	Not active[3]	(1–3) β-D-glucan terminated with mannitol	0
Laminaripentaose	Not active[3]	(1–3) β-D-glucan	0
Dextran T10, T20, T40, T70, T500	Not active[3]	α-D-glucan	0
Hyaluronic Acid	Not active[3]	(1–4) β-D-glucuronic acid; (1–3) β-N-acetyl-D-glucosamine	0
Cellulose	Not active[3]	(1–4) β-D-glucan	0
Starch	Not active[3]	α-D-glucan	0
Chitin[2]	Not active[3]	(1–4) β-N-acetyl-glucosamine	0
Cellobiose	Not active[3]	(1–4) β-D-glucan	0
Glucose	Not active[3]	—	0
Mannitol	Not active[3]	—	0
Endotoxin	10^{-3} to 10^{-4}	—	0.14

[1] Provided by Dr. A. Kakinuma.

[2] Tested as a suspension.

[3] All glycans were tested at concentrations ranging from 10^{-8}–10^{-1} mg/ml.

terminated with glucose and the latter with mannitol. Neither laminarin G nor M, in concentrations ranging from 10^{-8} to 1 mg/ml, activated the clotting system of *Limulus* amebocyte lysate (Table I). A small linear pentasaccharide (laminaripentaose), composed of (1–3) linked β-D-gluco-pyranosyl residues, also was ineffective in activating the proclotting enzyme (Table I).

We investigated the possibility that the activity of crude laminarin was due to a LPS contaminant. Laminarin lost all its activating capacity after being heated for 2 h at 180°C, a treatment which destroys the activity of LPS in the *Limulus* clotting system (Atkins and Heijn, 1965). More specifically, polymyxin B, which by binding to LPS (Cooperstock and Riegle, 1981) inhibits LPS-activation of proclotting enzyme activity (Nakamura and Levin, 1982a), also blocked activation by laminarin (Fig. 1). The β1,3-glucans of the laminarin preparation were unaffected by the polymyxin B treatment since we detected no significant differences in amidase or prophenoloxidase activation in hemocyte lysate from decapod crustaceans between polymyxin B-treated and untreated laminarin (Table II). Thus, we concluded that native glucans are unable to activate the *Limulus* clotting system, provided that they are free of endotoxin contamination.

Induction of clotting enzyme activity by the carboxymethylated glucan, CMPS

It has been reported that a derivatized glucan is capable of activating the clotting enzyme in lysates of amebocytes of the Japanese horseshoe crab, *Tachypleus tridentatus* (Kakinuma *et al.*, 1981; Morita *et al.*, 1981). The β1,3-glucan used by Kakinuma *et al.* (1981) and Morita *et al.* (1981) to induce activation of the horseshoe crab clotting

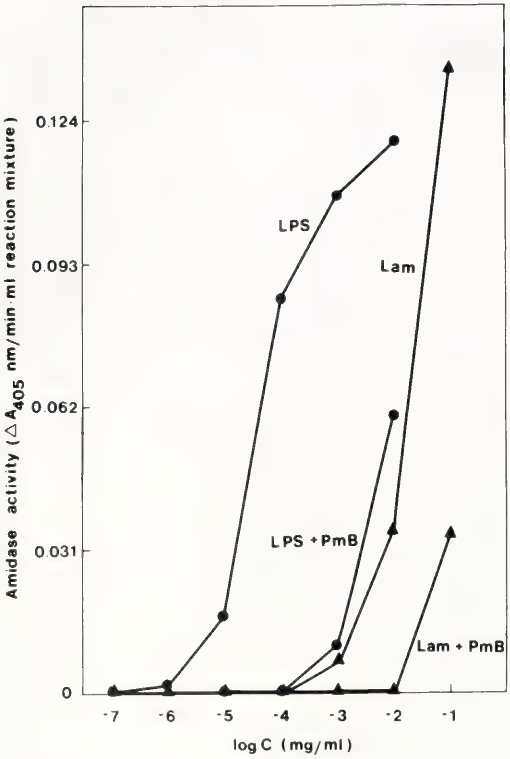

FIGURE 1. Effects of polymyxin B treatment of lipopolysaccharide (LPS) or laminarin (Lam) upon induction of clotting enzyme activity in *Limulus* amebocyte lysate. Various concentrations (C) of LPS or Lam were pretreated with polymyxin B (PmB) (100 U/ml) for 30 min before they were assayed for the induction of clotting enzyme activity, using the chromogenic peptide assay (see Materials and Methods).

system is a carboxymethylated curdlan (CMPS), with a degree of polymerization of approximately 500. In our hands, this CMPS also activated proclotting enzyme and generated clotting enzyme activity (Table I), which resulted in subsequent gelation of *Limulus* amebocyte lysate. Polymyxin B (final concentration, 1000 U/ml) did not affect CMPS-induced activation of proclotting enzyme activity, suggesting that activation was not due to endotoxin contamination (data not shown). Activation showed a curious dependence upon dosage, with inhibition occurring at CMPS concentrations above 10^{-2} mg/ml (data not shown) as also has been reported by Kakinuma *et al.* (1981). At these high concentrations (greater than 10^{-2} mg/ml), the CMPS preparation caused the immediate formation of a precipitate in *Limulus* lysate which could not be redissolved in 1% NaCl or distilled water. The supernatant prepared by removing this precipitate still contained endotoxin-inducible hydrolyzing activity towards Ac-Ile-Glu-Gly-Arg-pNA and coagulogen (Table III). This indicates that high concentrations of CMPS did not inactivate or precipitate critical components in the "endotoxin pathway" of the *Limulus* lysate clotting system or precipitate the coagulogen. Thus, based on these observations, it is suggested that there are two separate pathways in the clotting system of Limulidae, one activated by endotoxin and the other by CMPS. Consistent with this suggestion, different lysate preparations exhibited different sensitivities for CMPS, which did not correlate with their sensitivities to endotoxin (Table

TABLE II

Effect of polymyxin B treatment of a β1,3-glucan (laminarin G) upon activation of an endogenous proteinase and prophenoloxidase in decapod hemocyte lysates

Treatment of hemocyte lysate			
β1,3-glucan (mg/ml)	Polymyxin B (units/ml)	Amidase activity[1] (ΔA_{405} nm/min/ml)	Phenoloxidase activity[2] (ΔA_{490} nm/min/ml)
0.025	100	—	0.028
0.025	0	—	0.026
0.01	1000	0.036	—
0.01	100	0.039	—
0.01	0	0.042	—
0.0025	100	—	0.018
0.0025	0	—	0.017
0.001	1000	0.018	—
0.001	0	0.025	—
0	100	0.003	0.009
0	0	0.004	0.009

[1] Laminarin G (a β1,3-glucan) was treated with polymyxin B for 15 min at 20°C. Polymyxin B treated or untreated laminarin G then was assayed for capacity to activate serine proteases in the hemocyte lysate of the crayfish, *Astacus astacus*, by preincubating 100 μl hemocyte lysate with 100 μl treated or untreated laminarin G for 10 min at 20°C. After the preincubation, 100 μl of this mixture was added to 400 μl 0.01 *M* Tris-HCl buffer, pH 8.0 and 100 μl 1.5 m*M* chromogenic peptide (Bz-Ile-Glu-(γ-0-piperidyl)-Gly-Arg-pNA), and incubated at 37°C for 30 min. To terminate the reaction, 100 μl 50% acetic acid was added and then absorbance at 405 nm was measured.

[2] Laminarin (100 μl) was pretreated with polymyxin B (final concentration, 100 U/ml) for 2 h. It then was incubated with 100 μl of hemocyte lysate from the crab, *Cancer borealis*, and 100 μl of 250 m*M* $MgCl_2$ for 15 min, followed by incubation with 100 μl of L-dopa (3 g/l). Phenoloxidase activity was measured after 20 min incubation with L-dopa at 20°C (see Materials and Methods). Water was substituted for laminarin to ascertain that polymyxin B did not generate phenoloxidase activity.

IV). It is noteworthy that enzymatic activity generated by CMPS was only 15–42% of that produced by endotoxin (Tables I and IV).

Exocytosis of the Limulus *blood cell (amebocyte)*

In the unchallenged animal, the entire coagulation system of *Limulus* is contained within the secretory granules of the blood cells (Mürer *et al.*, 1975). The first event

TABLE III

Endotoxin activation and gelation of CMPS-inhibited Limulus *amebocyte lysate*[1]

Treatment of CMPS inhibited lysate	Gelation	Amidase activity (ΔA_{405} nm/min/ ml reaction mixture)
Endotoxin (10^{-4} mg/ml)	Yes	0.11
CMPS (10^{-2} mg/ml)	No	0

[1] CMPS (1 mg/ml) was incubated with an equal volume of amebocyte lysate for 1 h at 20°C. The mixture then was centrifuged for 20 min at 27,500 × *g* (6°C) to remove the precipitate which had formed, and the resulting supernatant tested for the ability to support production of hydrolyzing activity towards S-2423 or for the presence of clotting enzyme, following addition of endotoxin or additional CMPS. Control preparations of lysate caused to gel by the addition of 10^{-4} mg/ml endotoxin (no CMPS present) contained 0.14 units of amidase activity.

TABLE IV

Comparison of LPS or CMPS activation of different batches of Limulus *amebocyte lysate*[1]

	Amidase activity (ΔA_{405} nm/min/ml reaction mixture)		
	61–80[3]	64–80	156–81
LPS (10^{-4} mg/ml)[2]	0.136	0.098	0.139
CMPS (10^{-4} mg/ml)	0.020	0.033	0.059

[1] LPS or CMPS-generated clotting enzyme activity in different batches of *Limulus* amebocyte lysates was measured using the chromogenic peptide assay (see Materials and Methods).

[2] Concentrations of LPS and CMPS of 10^{-4} mg/ml were selected because CMPS was found to be inhibitory at higher concentrations.

[3] Numbers refer to batch number of amebocyte lysate.

necessary for coagulation to occur is the release of the enzymes and coagulogen, an event that occurs by exocytotic degranulation (Dumont *et al.*, 1966; Ornberg and Reese, 1981; Armstrong and Rickles, 1982). Degranulation of washed amebocytes maintained in observation chambers can be triggered by exposure to specific secretagogs dissolved in 3% NaCl. For example, degranulation of a monolayer of amebocytes occurred within 3–10 min following exposure to 10 μg/ml of bacterial lipopolysaccharide (LPS) (Fig. 2a, b), as observed previously by Armstrong and Rickles (1982). In this system washed amebocytes were nonresponsive to high concentrations (100 μg/ml, 30–60 min) of zymosan, CMPS (Fig. 2c), and two purified preparations of β1,3-glucans (laminarin G and M). After a 60 min exposure to the purified β1,3-glucan preparations, the cells were still capable of degranulating when exposed to LPS (10 μg/ml). Different amebocyte preparations varied in their response to laminarin; at high concentrations (100 μg/ml), some preparations degranulated whereas others did not. We interpret the positive responses that were occasionally observed with laminarin as resulting from the presence of LPS in this material (see Fig. 1). Pertinently, it has previously been demonstrated (Armstrong and Rickles, 1982) that some preparations of *Limulus* amebocytes are more sensitive than others to low doses of LPS (1 μg/ml). Apparently, the intact washed amebocyte does not recognize the β1,3-glucans as secretagogs, which is in contrast to the situation in crustaceans in which the semi-granular cells respond by exocytosis if challenged with β1,3-glucans (Johansson and Söderhäll, 1985).

Phenoloxidase activity in Limulus *blood*

The prophenoloxidase activating system of crustaceans and insects is important in the killing of invading parasites and microbes (Salt, 1970; Söderhäll, 1982) and in tanning of the cuticle after molting and during wound healing (Neville, 1975). The system can be activated by β1,3-glucans (Unestam and Söderhäll, 1977; Ashida, 1981; Ashida *et al.*, 1983; Smith and Söderhäll, 1983a; Söderhäll, 1983; Leonard *et al.*, 1985) and by bacterial products (Pye, 1974; Ashida *et al.*, 1983; Söderhäll and Häll, 1984). This enzyme system, so important in cellular defense in other arthropods, could not be detected by the standard biochemical tests in *Limulus* (Table V). Its absence also is suggested by the failure of aggregated preparations of living, extravasated amebocytes to melanize, even after maintenance for several days in organ culture (Fig. 3).

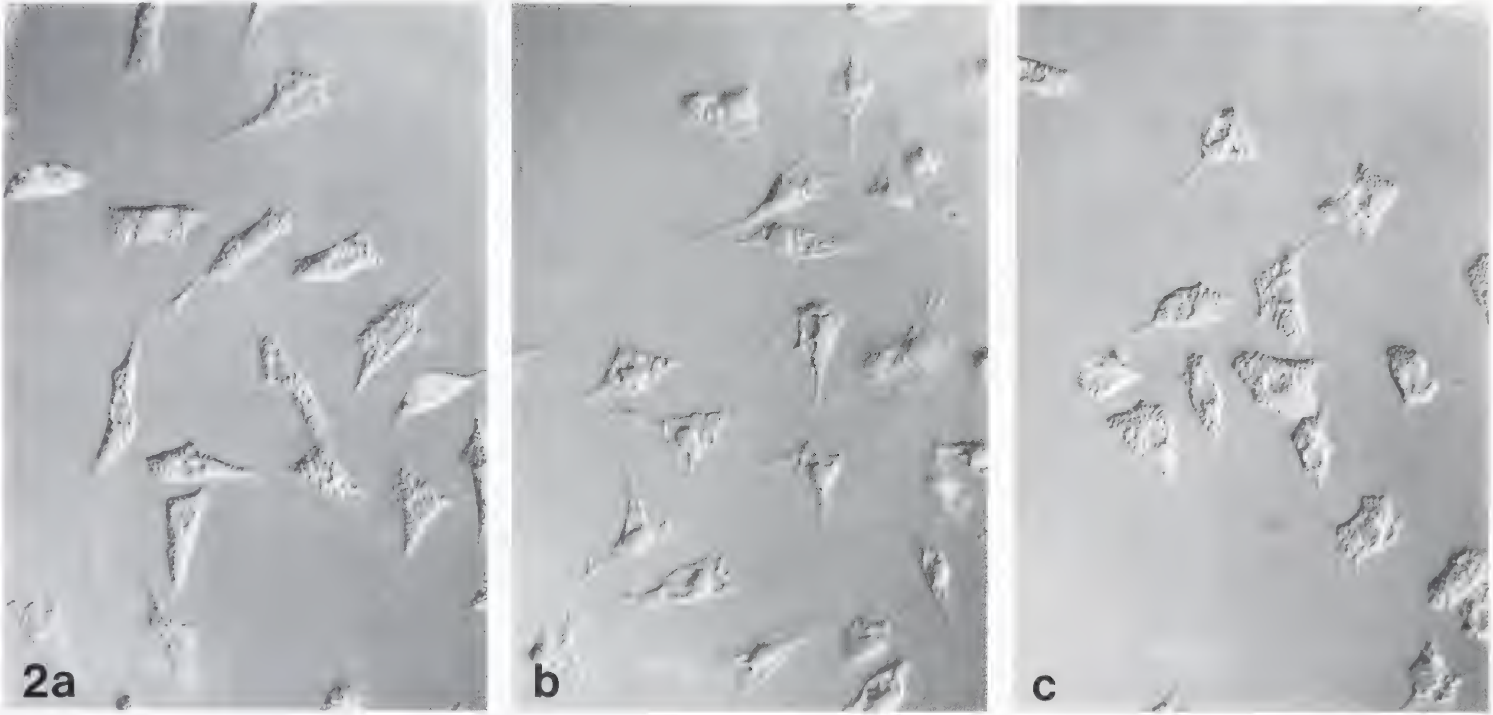

FIGURE 2. The ability of potential secretagogs to induce exocytosis of *Limulus* amebocytes can be studied by differential interference contrast microscopic examination of cells adherent to microscope coverglasses. The agent to be studied, in this case bacterial lipopolysaccharide (10 μg/ml), was dissolved in 3% NaCl and perfused beneath the coverglass. In Figure 2a, taken 1 min after exposure of the cells, the amebocytes are still fully granular (the granules are the refractile, oval bodies in the cytoplasm). Degranulation occurred at 4 min and by 5 min (Fig. 2b) the cells had completed exocytotic release of the granules. The glucan CMPS did not induce exocytosis of washed amebocytes at a concentration of 100 μg/ml. Time of exposure: 47 min (Fig. 2c). Similarly, zymosan and laminarins G and M were without effect (not shown). 470×.

TABLE V

Assays for phenoloxidase activity in Limulus *hemolymph*

Treatment of *Limulus* amebocyte lysate (LAL)	Phenoloxidase activity[1] ΔA_{490} nm/60 min ml reaction mixture
LAL	0
LAL + trypsin (0.25 mg/ml)	0
LAL + $CaCl_2$ (10 m*M*)	0
LAL + $CaCl_2$ (10 m*M*) + trypsin (0.25 mg/ml)	0
LAL + $MgCl_2$ (50 m*M*)	0
LAL + $MgCl_2$ (50 m*M*) + trypsin (0.25 mg/ml)	0
LAL + $CaCl_2$ (10 m*M*) + $MgCl_2$ (50 m*M*) + trypsin (0.25 mg/ml)	0
Limulus plasma	0
Limulus plasma + trypsin	0

[1] Enzyme activity determined as detailed in Materials and Methods.

DISCUSSION

Limulus, like many large arthropods, is relatively long-lived, requiring 9–12 years to reach maturity (Shuster, 1950, 1954), and with a maximum lifespan estimated to be in excess of 14–19 years (Ropes, 1961). One requirement for such longevity is the ability to defend against potentially pathogenic microbes (for review see Armstrong, 1985). The cuticle and epidermis certainly form important barriers to penetration. The one well-studied internal defense mechanism in *Limulus* is activation of the clotting system by bacterial endotoxin (Bang, 1956, 1979; Shirodkar *et al.,* 1960; Levin and Bang, 1964a, 1968; Levin, 1967; Stagner and Redmond, 1975). Other arthropods

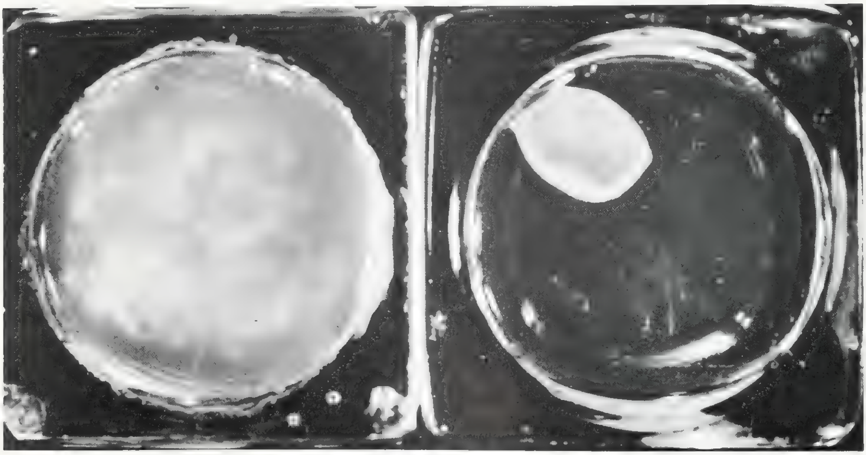

FIGURE 3. Amebocyte tissue can be prepared for study by collecting blood under aseptic conditions in embryo watchglasses. After removal from the animal, the blood cells settle and aggregate into a tissue-like mass that, after an extended period *in vitro,* undergoes contraction. In right-hand watchglass, it is 1 day old. The amebocyte tissue mass has contracted into the compact, white, button-like mass in the upper left of the watchglass. Even at 1 or more days after preparation, the aggregated mass of blood cells is white in color, with no sign of melanization. The fluid medium was *Limulus* plasma.

also respond to endotoxin by degranulation of certain classes of granular blood cells (Johansson and Söderhäll, 1985) and activation of the prophenoloxidase activating system (Söderhäll and Häll, 1984). In both crustaceans and *Limulus,* endotoxin apparently serves as an important humoral signal for the activation of appropriate host defense systems in the presence of gram-negative bacteria.

Fungi represent another important class of microbial pathogens for arthropods (Johnson, 1970; Sindermann, 1970). The β1,3-glucans, which are components of the cell walls of all fungi except the mucorales (Bartnickii-Garcia, 1972), serve as humoral activators of the defense systems of crustacean (Unestam and Söderhäll, 1977; Söderhäll, 1981; Smith and Söderhäll, 1983a, b) and insect (Pye, 1974; Ashida *et al.*, 1983; Leonard *et al.*, 1985) blood cells. These compounds, like endotoxin, stimulate blood cell clumping (Smith *et al.*, 1984) and exocytosis (Smith and Söderhäll, 1983b) and activation of the prophenoloxidase system (Söderhäll and Unestam, 1979; Ashida, 1981; Söderhäll, 1982; Ashida and Söderhäll, 1984).

The purpose of the present study has been to investigate the possibility that similar recognition-response patterns to fungal cell wall components are displayed by the blood cells of *Limulus,* as a representative of the chelicerate arthropods. Our results indicate that whole fungal cell walls (zymosan) and the naturally occurring β1,3-glucans are not recognized by the clotting system of *Limulus,* confirming previous reports that extracts from a variety of yeasts fail to gel *Limulus* amebocyte lysate (Cutler *et al.*, 1972; Jorgensen and Smith, 1973; Wildfeuer *et al.*, 1975). Although the artificially derivatized β1,3-glucan, CMPS, did trigger coagulation of the isolated clotting system, endotoxin-free preparations of naturally occurring β1,3-glucans were inactive. The production of a precipitate following exposure of *Limulus* amebocyte lysates to high concentrations of CMPS, which did not occur with any of the other tested glucans, suggests that the reaction between CMPS and horseshoe crab lysate may be complex. The observation that the maximal enzymatic activity generated by CMPS was only 15–42% of that produced by LPS is difficult to rationalize with the notion that both activate the same enzyme cascade system. In sum, the biological significance and underlying mechanisms of activation of the clotting system by CMPS are unclear. In addition, CMPS, zymosan, and all of the endotoxin-free natural β1,3-glucan preparations that were tested did not provoke exocytosis of isolated *Limulus* blood cells. Since, in the unchallenged animal the entire clotting system is contained within the exocytotic granules of the blood cells, and, under natural conditions, must be released by exocytosis to become active (Mürer *et al.*, 1975), this latter observation suggests that any naturally occurring β1,3-glucans that might be structural and functional homologues to CMPS would be unlikely to play a functional role in defense because CMPS does not trigger the release reaction.

A second interesting observation made during the present study is that *Limulus* blood apparently lacks prophenoloxidase activity. This system is widely distributed in the arthropods and serves several important roles in defense (Ashida, 1971, 1981; Söderhäll, 1982; Ashida *et al.*, 1983; Ashida and Söderhäll, 1984; Söderhäll and Häll, 1984; Leonard *et al.*, 1985). The biochemical assay conventionally used to assay phenoloxidase activity in the blood of insects and crustaceans consistently failed to reveal activity in the blood or lysates of the blood cells of *Limulus.* Also, isolated blood cells failed to melanize following bleeding, consistent with previous observations that melanization does not occur during wound healing in *Limulus* (Bursey, 1977).

In summary, the blood-based defense systems of *Limulus* appear to lack certain characteristics seen in insects and crustaceans, namely the ability to respond to fungi or fungal cell wall glucans and the ability to activate phenoloxidase in response to bacterial or fungal cell wall products. It will be of interest to discover whether these deficits are present in other chelicerate arthropods. It also will be important to elucidate the mechanisms that confer resistance of *Limulus* to potentially pathogenic fungi.

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ADHESIVE INTERACTIONS BETWEEN THE TUBE FEET OF A STARFISH, *LEPTASTERIAS HEXACTIS*, AND SUBSTRATA

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ABSTRACT

The tube feet of *Leptasterias hexactis* adhere to and release from substrata by chemical interactions. In our laboratory these podia adhered to substrata coated with the ubiquetous anionic saccharide films produced by marine bacteria. Podia also attached to moderately anionic glass, but not to uncharged surfaces. The adhesive epithelia of tube feet labeled heavily with ruthenium red, indicating they were anionic. Tube feet secreted footprint films that bound crystal violet, a cationic dye. Trypsin removed the films. Adhesion to marine surfaces was prevented by 300 units/ml of heparin, a glycosaminoglycan (GAG) that may have competitively inhibited the glue from binding exosaccharide marine films. Lectins that bind bacterial exosaccharides did not inhibit attachment. We propose that tube-foot attachments are nonspecific ionic interactions established by secreted proteinaceous films and released when secreted GAG's compete with the tube-foot epithelium for sites on the film. This system agrees with the duo-gland model for adhesion and deadhesion.

INTRODUCTION

The tube feet of echinoderms, and asteroids in particular, are traditionally viewed as miniature suction cups that are aided in their attachment to substrata by adhesive secretions (Smith, 1947; Thomas and Hermans, in press). The functional morphology of the tube feet of *Asterias rubens* was analyzed by Smith (1947) who developed a model that describes how tube feet may function as suction cups. This model ignored chemical ("mucus") adhesion although the work of Paine (1926) was cited. Paine (1926) had concluded that roughly 44% of the adhesive forces in the tube feet of *A. vulgaris* comes from chemical adhesion. The question of how each tube foot is chemically attached and detached during the pedal locomotory cycle has been raised and needs to be resolved (Hermans, 1983).

During preliminary observations for this study a *Leptasterias hexactis* in our laboratory adhered to a somewhat corroded, well-used stainless steel plankton screen (10 mesh/mm). Although it was not entirely clean, the mesh was open, and the starfish adhered so well that tube feet broke off and remained on the net when the animal was pulled free. The average diameter of the adhesive discs of *L. hexactis* is 1.0 mm. It would have been impossible for the podia to have used suction for attachment to the screen.

Anyone observing starfish in aquaria will note that the tube feet generally remain clean during the attachment/detachment/reattachment cycle in locomotion. Adherent material does not accumulate on the adhesive surfaces. If there is a chemical adhesive it is either torn away from the tube foot at each step or a chemical mechanism frees each podium of adherent material. Kerkut (1953) demonstrated that tube feet ordinarily

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are not torn from the substratum at each step during normal locomotion. He showed that the tube feet do not stick to or pull upon the substratum as they are lifted away. Electron micrographic studies have described cells that were claimed to secrete adhesives (Harrison and Philpott, 1966; Chaet, 1965), but Hermans (1983) suggested that the putative adhesive granules may be deadhesive or cleansing in function and that actual adhesive materials may be produced by other cells.

We agree with Roth (1983) that biological adhesion mediated by proteins binding carbohydrates may have evolved only once, and, observing that biological adhesive relationships are often temporary, we believe that duo-gland relationships, in which adhesiveness is modulated by secretions that promote adhesion or flow, are wide spread but not recognized in many cases (Hermans, 1983). The study of echinoderm tube feet will shed light on duo-gland, adhesion/flow or adhesion/detachment relationships generally. Therefore we have examined some chemical interactions between the tube feet of *Leptasterias* and various substrata under varied conditions.

MATERIALS AND METHODS

The starfish used in this study were members of the genus *Leptasterias,* small six-rayed forcipulates, collected from the mid-intertidal zone at Shell Beach, Sonoma County, California. They were maintained in marine aquaria at 12°C. The current taxonomy of *Leptasterias* is unclear (Fisher, 1930; Chia, 1966; Sutton, 1975). The animals used in this study were collected from a single population on one large boulder, and a few nearby rocks. The specimens ranged from 2 to 5 cm diameter and conformed most closely to Fisher's (1930) description of the polymorphic species *Leptasterias hexactis* (Stimpson). These starfish were chosen because of their availability, convenient size, and because they are similar to a variety of the most common species of starfish found in temperate waters.

Tube feet were collected by pulling *Leptasterias* from rocks. The tube feet that tore from the sea star by remaining attached to the rock were fixed in glutaraldehyde-ruthenium red, and postfixed in osmium tetroxide-ruthenium red, according to Mellonig (1976). Some starfish were allowed to attach to *Phyllospadix,* a plant normally found in the habitat of *Leptasterias,* and the tube feet were severed and fixed while still attached to the plant. These were examined microscopically using the methods of Thomas and Hermans (in press).

Footprints left by walking starfish were obtained by allowing starfish to walk across clean glass slides (Scientific Products). These were stained with 0.005% aqueous crystal violet and examined with a Leitz compound microscope equipped with an Ortholux camera.

The adhesiveness of tube feet to various substrata was determined in the following manner. Substrata were placed in the bottoms of finger bowls (4½ inch diameter glass culture dishes from Carolina Biological Supply, Burlington, North Carolina 27215) and covered to a depth of about 0.75 cm (50 ml) of fresh seawater or other medium. The finger bowls had been cleaned with 1% acetic acid in 95% alcohol and rinsed in seawater before using. Starfish were added and allowed to attach to the substrata in the dishes. When an animal began to move in a particular direction, its forward progress was impeded by gentle, finger tip pressure against the advancing edge. Starfish that had attached to the substratum offered finger-tip resistance that was clearly tangible. Unattached starfish float free at the slightest touch. Only animals that adhered normally to surfaces in the aquaria before and after each experimental test were counted. Starfish were placed repeatedly on the experimental surfaces because the starfish exhibited considerable "free will." By "free will" we mean that attachment to surfaces does not appear to be governed by one simple reflex. Starfish often fail to adhere to substrata upon which they have previously demonstrated the ability to attach. For instance, excessive handling or attempting to harness a starfish to a tensiometer inhibits attachment behavior. Stroking the backs (aboral surfaces) of the animals with light finger pressure, however, was found to stimulate indifferent animals to increase adhesion on suitable substrata. It was not possible to stimulate any adhesion on certain substrata (see Results).

The surfaces tested were teflon tape, Parafilm, dental wax, polystyrene culture dishes, 4½" diameter glass culture dishes (Carolina Biological Supply, soda lime glass), rubber, glass microscope slides, Medcast epoxy resin (Ted Pella, Co.), and clean glass microscope slides coated with sebum from otherwise clean human skin.

The distribution of negatively charged sites on substrata was visible by staining with 0.005% aqueous crystal violet for one minute and rinsing with deionized water. Crystal violet binds quantitatively to negatively charged sites (Maroudas, 1975). A violet color imparted to the surface gave a clear visual impression of negatively charged sites on surfaces, and the relative intensity of the color indicated the relative numbers of negative sites per unit area.

The increase in the relative numbers of negatively charged sites per unit area on the surfaces of glass culture dishes maintained in fresh aerated seawater for periods up to several months was measured in the following way. The culture dishes were drained, and the inside bottom of each dish was stained with 20 ml of 0.005% aqueous crystal violet for two minutes and rinsed with 50 ml of deionized water. The crystal violet that had adhered to the surface was eluted in 20 ml of 95% ethanol with gentle agitation for two minutes. The absorbance of the alcoholic crystal violet elutant was measured at 593 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer. The absorbance of each sample is proportional to the number of negatively charged sites per unit area of culture dish bottom.

The effects of the divalent cations Mg and Ca on the adhesion of tube feet to surfaces were investigated using solutions of 0.01 *M* and 0.005 *M* EGTA or EDTA (disodium form) in seawater, pH 7.4, with the osmolarity adjusted to that of Northern California seawater (950 mOsm). Normal seawater contains 0.04 parts calcium ion per 100 parts seawater (0.01 *M*) and slightly less than twice that amount of magnesium ion, both relative to the chlorinity of the seawater (Sverdrup *et al.*, 1942). Each mole of EGTA binds two moles of calcium and each mole of EDTA binds one mole of divalent cations nonspecifically. The surfaces tested were clean glass slides that bore inherent negative surface charges (determined by crystal violet staining) and natural marine films formed on the clean culture dishes that had been soaked in fresh, aerated seawater at 12°C for six weeks. Five starfish were placed on one of the experimental surfaces in a culture dish containing normal seawater and allowed to attach. The seawater was poured off and enough EDTA/seawater solution was added to cover all the tube feet. The animals were gently dislodged from their attachment and given one minute to re-attach. The solution was poured off and replaced with fresh seawater, and the animals were given another one minute to re-attach.

The relationship between adhesion and the layer of calcium cations that accumulate near negatively charged substrata in solution (Adamson, 1982; Fletcher *et al.*, 1980) was studied by allowing culture dishes to equilibrate for 20 minutes with 50 ml of 0.2 *M* EGTA in normal seawater. The chelating solution was poured off and the bowl was rapidly rinsed and refilled with 50 ml of normal seawater. Starfish were quickly added and their ability to adhere was recorded. The control for this test consisted of allowing the starfish to attach to the test dish before the substratum was chelated. This established the ability of the sample animals to attach firmly to the unchelated marine film.

The influence of heparin on the binding of tube feet to clean anionic glass slides was determined. These tests were conducted at Shell Beach and all solutions were kept at seawater temperature. Filtered natural seawater was used throughout. Fresh starfish were used for each test (n = 5). Thirty milliliters of filtered seawater were added to culture dishes containing glass slides (Scientific Products). This was enough seawater to cover the tube feet, but not the aboral disc of the starfish. Starfish were allowed one minute to adhere; only adherent starfish were used for the tests. The seawater was then replaced with 30 ml of heparin/seawater solution. After five minutes any still-adherent starfish were gently dislodged and allowed ten minutes to re-attach. The heparin solution was then replaced with natural seawater and the starfish were allowed ten minutes to re-attach. The test solutions consisted of natural filtered seawater and heparin sodium salt (Sigma Chemical Company) at concentrations of 300, 150, and 75 units/ml. At these concentrations the heparin had no effect on the pH and added not more than 0.4 mOsm to the seawater.

The possibility that lectins play a role in the adhesion of tube feet to naturally occurring marine films was investigated using films that developed on culture dishes as described above and 0.05 *M* solutions of those sugars, in seawater, that are known to be major components of gram-negative bacterial films. Some of these sugars have been shown to inhibit lectin binding in other systems (Kirchman *et al.*, 1982, 1984; Sutherland, 1972, 1980; Brown *et al.*, 1969 and Williams *et al.*, 1979). The monosaccharides tested are listed in Table I.

To gain some information on the chemical composition of the glue produced by tube feet a study was made of the effects of various chelating agents (EDTA and EGTA) and enzymes (trypsin, chymotrypsin, alpha amylase, and beta amylase) on footprints left on glass slides by starfish that had adhered to glass slides and were then gently removed. The slides with the footprints on them were immersed in Coplin jars of either test or buffer solutions at 22°C for one hour, and then stained with either crystal violet or aqueous carmine. The following test solutions were used: aqueous EDTA (0.2 *M*), saturated aqueous EGTA, 0.5 mg/ml trypsin or chymotrypsin in 0.2 *M* phosphate buffer (pH 8.0), alpha amylase in pH 5.5 phosphate buffer, and beta amylase in pH 3.5 acetate buffer. The enzymes were purchased from Boehringer Mannheim Biochemicals.

RESULTS

The surfaces of the *Phyllospadix*, to which *Lepatasterias* had adhered when fixed, was covered by a bacterial film 1 μm thick containing rod-shaped and spherical gram negative bacteria 300 nm in diameter embedded in a fibrillar matrix (Fig. 1). Between

TABLE I

Monosaccharides	
αL(-)-Fucose	D(+)-Glucosamine
D(+)-Galactose	3-0-α-methyl glucose
2-Deoxy-D-Galactose	D-Mannose
D-Galactosamine	α-Methyl-D-mannose
D-Glucose	D-Ribose
1-Deoxy-D-Glucose	

the bacterial layer and the adhesive surface of the tube feet there was a descrete layer of amorphous substance that averaged 2.2 μm in thickness.

The adhesive surfaces of the tube feet bound ruthenium red strongly, forming an electron-dense coat that obscured the glycocalyx and microvilli (Fig. 2).

When starfish walked across clean glass microscope slides they left footprints that stained heavily with crystal violet (Fig. 3). When the staining and rinsing were done gently these footprints retained thick films of material that stained in a reticular pattern. These films were the same diameter as the adhesive epithelium on each tube foot and corresponded to the deposits seen beneath attached podia in electron micrographs. The reticular pattern in each film consisted of heavily stained boundaries that separated larger areas that stained more lightly than the boundaries, but more heavily than the surrounding glass slide. The clean glass microscope slides to which tube feet adhered stained evenly and lightly with crystal violet.

The tube feet did not adhere equally well to all surfaces. There was a positive correlation between adhesivity, moderate negativity (crystal violet binding) and hydrophilic (water-drop tests) surfaces. These results are summarized in Table II.

Significant increases in the density of negatively charged sites developed as clean glass was soaked in fresh seawater (Fig. 4).

Starfish attached to previously uncharged and non-adherent glass surfaces after they acquired natural marine films. But if the films became too thick, they became slimy, and starfish could no longer adhere. This occurred when one batch of glass was kept for several months in a marine aquarium that had been inoculated with bacteria on rocks from a quiet region of Bodega Bay. The dishes became heavily coated with a negatively charged slime of bacteria, diatoms, algae, and other small organisms. These conditions could not have developed on the outer coast where *Leptasterias* is found, because wave action and grazing animals would have reduced the film before it approached the thick slimy non-adherent stage. When these heavily coated glass surfaces were superficially cleaned with a strong stream of water or gentle wiping, so that they were still coated but no longer slimy, the starfish adhered well.

Starfish adhered firmly in culture dishes that had developed marine thin films in normal seawater before and after immersion of the tube feet in 0.01 *M* or 0.005 *M* solutions of EDTA in seawater, but did not adhere to the same surfaces while in this solution. Podia that had attached before ordinary seawater was replaced by seawater containing EDTA did not spontaneously detach, yet once their hold on the bottom of the glass dishes had been broken they did not reattach in the presence of EDTA. The podia moved normally in the chelating agent for only about a minute. If left in the solution for longer than three minutes they gradually became noticeably less active.

The results of the same tests using 0.01 *M* and 0.05 *M* solutions of EGTA in seawater were consistant with those for EDTA. Starfish that were attached did not detach spontaneously when the EGTA was added; after being dislodged they did not re-attach until after the EGTA solution was replaced with normal seawater. The tube feet retained normal levels of activity during the time course of these experiments, but if left in the chelating agent for long periods they became inactive. Nevertheless, they still adhered well in normal seawater before and after being in the solution of chelating agent.

Tube feet failed to adhere to clean glass slides in 0.01 *M* EGTA, but they did adhere lightly in 0.005 *M* EGTA and were normally adherent in the control tests with ordinary seawater before and after the experimental solutions. Under control conditions the tube feet left footprints on the glass slides. They left some footprints when they were in 0.005 *M* EGTA solution. They did not deposit footprints on the glass in solutions of 0.01 *M* EGTA.

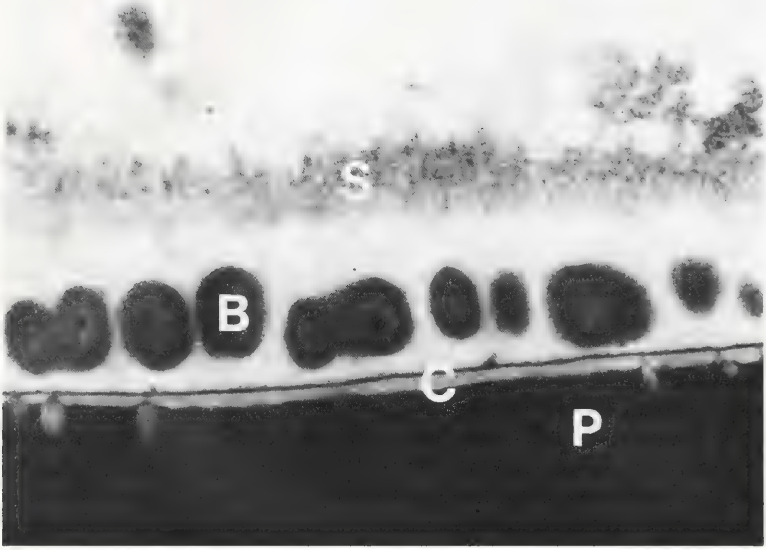

FIGURE 1. Gram-negative bacteria coating the surface of *Phyllospadix*. B = bacterium; C = cuticle of *Phyllospadix*; P = *Phyllospadix*; S = tannic-acid labeled bacterial exosaccharide film. 30,000×.

Starfish in normal seawater adhered poorly to substrata coated with marine films that were chelated with 0.2 *M* EGTA for 20 minutes immediately before starfish were placed upon them.

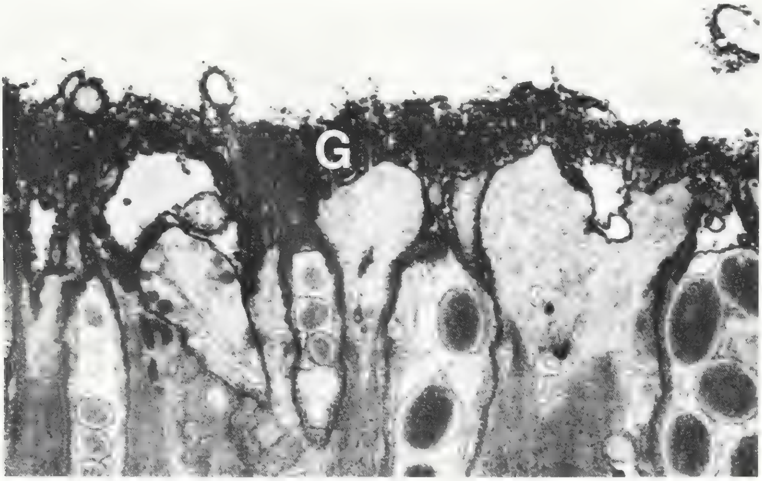

FIGURE 2. Electron micrograph of section through adhesive surface of tube-foot epithelium showing the apical glycocalyx (G) heavily labeled with ruthenium red. 10,000×.

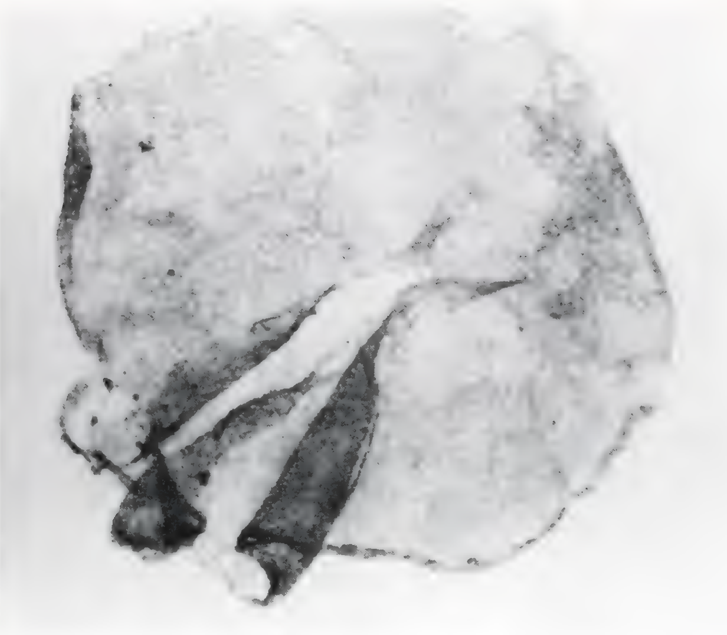

FIGURE 3. Light micrograph of adherent film (footprint) left on glass slide by walking starfish and stained with crystal violet. 40×.

Footprints that were soaked in chelating solutions for one hour appeared entirely normal when stained with crystal violet and examined microscopically. Neither EDTA nor EGTA disrupted the footprint films.

Heparin at a concentration of 300 units/ml prevented starfish attachment. It also caused the release of previously attached animals within five minutes. At 150 units/ml only three out of five animals spontaneously released, and detached animals could form weak bonds to the substratum within five minutes. After five minutes in a solution of 75 units/ml all animals remained attached, and within five minutes after

TABLE II

Ability of tube feet to bind various substrata compared to the anionic charges (crystal-violet binding) and hydrophobicity (water-drop tests) of those surfaces

Substratum	Tube-foot adhesion	Crystal-violet stain	Water-drop test
Glass slides	Firm	Yes	Hydrophilic
Rubber	Firm	Yes	Hydrophilic
Marine film on glass dish	Firm	Yes	Hydrophilic
Clean glass dish	None	No	Hydrophobic
Teflon tape	Weak	Uneven	Uneven
Polystyrene	Weak	Uneven	Uneven
Parafin	None	No	Hydrophobic
Dental wax	None	No	Hydrophobic
Epoxy resin	None	No	Hydrophobic
Sebum	None	Yes	Intermediate

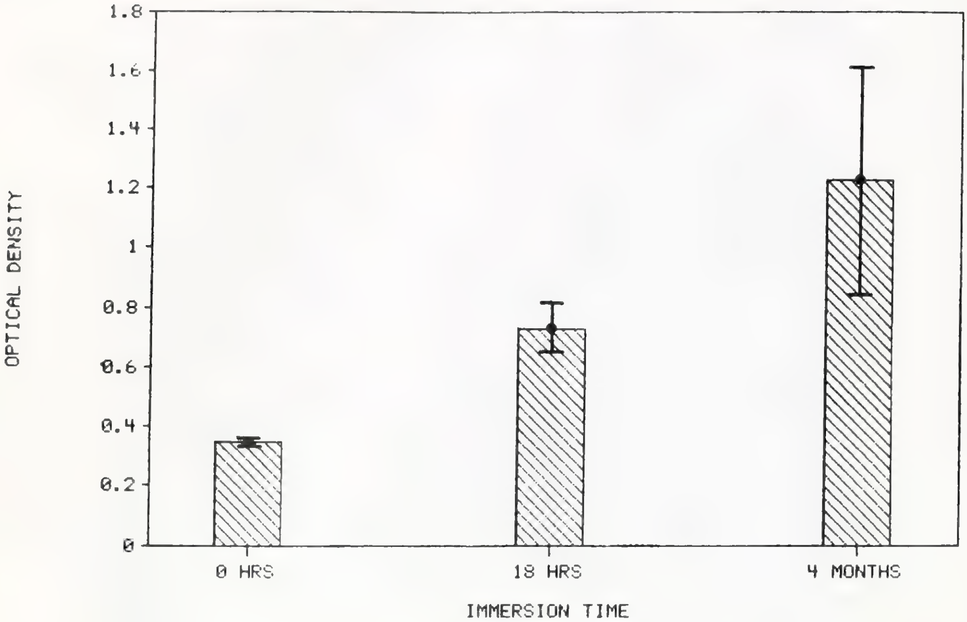

FIGURE 4. Relative amounts of crystal violet staining insides of bottoms of glass culture dishes per unit surface area. Average absorbance of 0.354 (absorbance units at a wave length of 593 nm, S.D. = 0.009) on recently cleaned glass; average absorbance of 0.731 (S.D. = 0.08 on glass that had been soaked in aerated fresh seawater for 18 hours); average absorbance of 1.2288 (S.D. = 0.386) on glass that had been soaked in aerated fresh seawater for four months.

being dislodged there was some re-attachment. All starfish were able to re-attach firmly within five minutes after replacement of the heparin solutions with normal filtered seawater.

Trypsin removed all traces of footprints from glass slides. Examination under both the dissecting microscope and the Leitz compound microscope revealed clean slides, devoid of all matter, and crystal violet did not bind footprint sites. Control prints soaked in the same buffer as the trypsin solutions were completely intact and stained well.

Prints treated with amylases were disrupted but remained on the slides, and tube feet which were severed from the starfish and remained attached to the slides were still present after the amylase digestions. Footprints treated with beta amylase appeared less disrupted than those treated with alpha amylase.

None of the monosaccharides used as potential competitive inhibitors of lectins prevented tube feet from adhering.

DISCUSSION

The epithelium on the adhesive surface of the tube feet of *Leptasterias* contain three types of cells: adhesive cells, large-granule secreting cells, and monociliated cells. The adhesive cells contain small dense granules and have broad distal surfaces covered with microvilli that bear numerous fine filaments at their tips. These cells also contain large bundles of intermediate filaments that connect the adhesive surface of each cell with the tension-bearing network of collagen between and beneath the cellular elements of the adhesive epithelium. Cells that secrete large granules are arranged alternately with the adhesive cells. The large granules are released through narrow collars of microvilli that form secretory channels between the glycocalyces of the neighboring adhesive cells and deliver the granules between those glycocalyces and the substrata to which they adhere. The monociliated cells scattered throughout the adhesive epithelium are probably sensory (Thomas and Hermans, in press).

In this study we have demonstrated that the glycocalyx covering the adhesive surface of the tube foot stains heavily with ruthenium red. This fact indicates that the glycocalyx bears many negatively charged sites (Luft, 1971, 1976). Thus the entire distal surface of the tube foot is coated by a negatively charged surface which somehow attaches to substrata.

Tube feet, like cells *in vitro,* do not attach equally well to all substrata. In both cases the cells attach to charged substrata that have sufficient rigidity to support the tensions exerted on them (Maroudas, 1973, 1975). *Leptasterias* tube feet do not attach to uncharged surfaces (Parafilm, dental wax, epoxy resin, glass slides from Scientific Products) or to charged surfaces that are lacking in rigidity (sebum, very thick marine films). The water-drop test, which measures the contact angle of a drop of water placed on a substratum, is generally used as an index of hydrophobicity, but according to Maroudas (1973, 1975) this test is not sufficiently sensitive to detect small charged sites that are nevertheless adequately large to serve as points of attachment on otherwise hydrophobic surfaces. Maroudas (1973) found that small impurities provide enough charged sites for the attachment of cells to otherwise hydrophobic surfaces *in vitro.* The moderately anionic surfaces to which tube feet adhere may seem hydrophobic by water-drop standards when compared with highly charged surfaces, but their true characteristics are apparent when hydrophobic materials such as paraffin are included in the tests. This explains why tube feet were moderately adhesive with respect to polystyrene and teflon in spite of these surfaces' general hydrophobicity. They contain sufficient anionic sites, as revealed by crystal violet, to make adhesion possible. We also observed that glass surfaces, usually considered to have similar characteristics, sometimes differ significantly in surface charge. Some glass samples (cleaned culture dishes, Carolina Biological) frequently lacked sufficient charged sites for podia to attach (crystal violet test), but glass slides (Scientific Products) did have sufficient charge, and tube feet attach well on them. However, highly charged negative surfaces are repellent to tube feet, and may even be toxic (Sechler and Gunderson, 1974).

To understand the mechanisms by which starfish attach and detach, we must understand the characteristics of the moderately negative microbial films that coat all marine surfaces (Characklis, 1981; Neihof and Loeb, 1974). Brewer (1984) described such films as hydrophobic, but our own tests confirm that they are negatively charged and that bacteria can double (from 0.354 to 0.731 absorbance units, see results) the number of negatively charged sites on glass in less than a day. The films consist of macromolecules and bacteria that are deposited in a two-step process. When immersed in seawater, materials with differing wettabilities, surface tensions, and electrophoretic mobilities are instantaneously coated with an organic molecular layer, usually polysaccharides and glycoproteins (Characklis, 1981). Electropositive or strongly electronegative surfaces are unlikely to exist in natural seawater because they rapidly adsorb this dissolved organic material, and it imparts a characteristically moderate negative charge to the surfaces (Neihof and Loeb, 1974). The adsorbed organic layer conditions the surface with nutrients and provides a slightly negative surface charge. It then attracts motile marine bacteria, and they, as well as randomly arriving non-motile bacteria, attach to the molecular film (Marshall, 1974).

Irreversible bacterial attachment occurs when bacteria produce polysaccharide polymers that are acidic due to numerous uronic acid groups. These polysaccharide fibers utilize calcium, magnesium, and iron for intermolecular bonding that creates the polyanionic carbohydrate slime characteristic of marine surfaces (Characklis, 1981; Corpe, 1970, 1974). Many studies support the theory that bacterial films form the preferred substratum for the attachment of other marine organisms (Meadows and Williams, 1963; Bracato *et al.,* 1982; Kirchman *et al.,* 1982, 1984; Brewer, 1984).

In the case of *Phyllospadix* we have demonstrated that the substratum to which tube feet attach is a bacterial film coating the surface of the plant (Fig. 1). Since the glycocalyx on each podium and the bacterial film coating *Phyllospadix* are acidic in nature, the chemical adhesive material must be capable of cross-linking acidic surfaces. The numerous microvilli projecting from the adhesive cells provide structural reinforcement to the glue matrix as it coats the highly irregular surfaces found in marine environments.

Chemical attachment of the adhesive epithelium to a substratum occurs over the entire contact surface of the disc. Thomas and Hermans (in press) showed that the adhesive epithelium is morphologically uniform, and the evenly labeled footprints left by firmly attached starfish indicate that the epithelium is also functionally uniform. These complete footprints did not contain unstained regions such as those described by Smith (1947) for *Asterias.* However, the animals frequently used little attachment when crossing level surfaces in glass dishes. Under these circumstances the podia apparently serve as levers and struts (Kerkut, 1953) that propel the animal forward while expending little glue for actual attachment. The ability to regulate the release of glue is energetically advantageous and explains the high variability of adhesiveness observed in individual tube feet of the same size (Paine, 1926). It also provides for the fact that tube feet that manipulate eggs and larvae (Chia, 1968) can also adhere to substrata with a force that exceeds the tensile strength of the stems.

The footprints deposited by tube feet on glass slides are films with negatively charged surfaces, and they correspond in size and pattern to the adhesive epithelium of the podia. The broad areas in the footprints that stain lightly with crystal violet correspond to the large surfaces of the adhesive cells, and the narrow more darkly stained bands correspond to the pattern formed by the cells that secrete the large granules between the adhesive cells (Fig. 3).

The fact that footprint films stain with crystal violet suggests that at least one of the components is acidic. One explanation for this acidity is that the secreted glue might consist of glycosaminoglycans (GAG's) that use divalent cations to link acidic glycocalyces to acidic substrata. This model has been proposed for bacterial adhesion (Fletcher *et al.,* 1980). However, the footprint films left by walking tube feet were not disrupted by either the general divalent cation chelating agent EDTA or the calcium-chelating agent, EGTA. Assuming that EGTA had access to any calcium ions, and that electrostatic repulsion between the GAG's and EGTA did not inhibit the chelating activity, it appears that divalent cations are not essential for the integrity or adhesion of the footprint films. However, calcium ions are clearly important in the adhesive process of podia, especially the calcium-ion concentration that probably accumulates at negatively charged surfaces (see discussion of double electrical layers in Adamson, 1982). Tube feet in EGTA-chelated water initially retained their normal mobility, but could not attach to the substrata, and they adhered poorly even in normal seawater when the substrata had been chelated with EGTA. We conclude from this that calcium is not an essential element in the structure or function of podial glue, but that it is required by the exocytotic process of secretion. Since EGTA is quite specific for calcium ions, it is apparent that neither magnesium nor other divalent cations, which would have remained in the test solutions of EGTA, can substitute for calcium in the adhesive process.

There is an alternative model to explain the anionic content of footprint films. The footprints may be composed of a basic protein released from the small dense granules in the adhesive cells and coated with negatively charged polymers released from the cells secreting the large granules from the adhesive epithelium. Accordingly, basic proteins may directly link the negatively charged glycocalyces on the adhesive epithelium to negatively charged substrata, or more likely, either basic or amphoteric proteins could polymerize into filaments linking the tube feet to the substrata. The thick matrix seen in micrographs of the interface between attached tube feet and *Phyllospadix* support the polymer hypothesis (Thomas and Hermans, in press). With basic proteins forming the glue, detachment of tube feet could be accomplished by the release of glycosaminoglycans (GAG's) from the large granules. The GAG's would compete with the glycocalyx for sites on the glue, thus releasing the tube foot and leaving a footprint film consisting of glue coated with GAG's on the substratum. This releasing factor may be similar to the GAG heparin, which releases podial attachments at higher concentrations and prevents attachment at lower concentrations. The anatomy and distribution of large-granule producing cells is appropriate for the delivery of such a product to the interface between glycocalyx and glue.

This basic protein/GAG model for adhesion and release is supported by the fact that the most strongly negative parts of the footprint films correspond to the apices of the large-granule secreting cells and the fact that the comparable large granules in other species of starfish have been demonstrated to contain GAG's (acid mucopolysaccharides) (Harrison and Philpott, 1966; de Sousa Santos and Sasso, 1968). The fact that thorium stained only the outer matrix of the large granules (Harrison and Philpott, 1966) can be explained by the fact that thorium penetrates very poorly.

The fact that the carbohydrate-digesting enzymes, alpha amylase and beta amylase, failed to completely release footprint material from glass slides, whereas digestion with trypsin and chymotrypsin did, supports this model. Although the glue may contain complex carbohydrates not digested by amylase it is still probable that the protein moiety binds the films to glass. Whereas, most of the saccharides in footprint films may be from the releasing agent, a small amount may be associated with the adhesive protein, if it is similar to known extracellular adhesive glycoproteins such as fibronectin (Yamada, 1983a, b).

The inhibition of podial attachment by heparin lends further support to this model. Heparin is a highly acidic polysaccharide that binds to the charged amino residues of basic proteins. According to our model heparin may prevent podial attachment by binding to cationic sites on the proteinaceous glue thereby preventing the glue from attaching to the acidic groups of the substrate film.

Tube feet do not attach with lectins that bind the monosaccharides common to bacterial films. We might have expected lectins to play a role because some marine invertebrates do use lectins when attaching to substrata (Kirchman *et al.,* 1982). In the latter case, however, the larvae of sessile organisms are apparently being aided in selecting appropriate substrata for permanent attachment by the specificity of the lectins involved. A mobile starfish like *Leptasterias* would not be aided by such a specific attachment mechanism.

Suction has been regarded as the primary means of tube-foot adhesion. Paine (1926) concluded that 44% of podial attachment is contributed by glue and that the rest is from suction. This conclusion was based on several assumptions that are not valid with respect to *Leptasterias.* She assumed that podia adhere equally well to various substrata. Total adhesion was tested by allowing podia to attach to glass and the adhesiveness of glue was measured by allowing them to attach to the open ends of rubber tubes. We know nothing about the characteristics of either of these surfaces. Second, it was assumed that only the periphery of a sucker uses glue for adhesion, since this adhesion was measured by allowing podia feet to attach to the open ends of tubing. The hollow center of tubing not only prevented suction from forming, but also eliminated an unknown proportion of chemical attachment. Finally, Paine assumed that the tension exerted on an attached podium at the time of release is the maximum tension sustainable. This ignores the "free will" aspect of podial release. This "free will" made it very difficult for us to collect podia attached to pieces of *Phyllospadix* because more often than not, when the plant was gently pulled upon the attached podia released. Clearly, when a small portion of substratum shifts, the podia attached to it frequently release. This response explains the large range of results Paine (1926) reported. Furthermore, when well-attached starfish are pulled from substrata the stems of many tube feet break, leaving the discs and portions of stems attached to the substrata. Obviously the ability of a disc to adhere exceeds the tensile strength of the stem. Therefore, when Paine measured the tension sustained by podia at the time of release she could not possibly have obtained the maximum tension sustainable by the adhesive epithelium. From this we conclude that Paine (1926) and others have grossly underestimated the contribution of glue to podial attachment and have failed to see the need for a chemical detachment mechanism.

Although the tube feet of starfish may also use suction on solid surfaces, it appears to be a secondary adjunct to the adhesion established by glue. The attachment of podia fits the extended model of the "contact hypothesis" of cellular adhesion presented by Maroudas (1975). According to this theory secreted polymers form bridges between cell membranes and substrate surfaces, making close molecular contact with both. In the "bridging" model the two most important characteristics of substrata are rigidity and surface charge. Because podia attach consistently to rigid, moderately negative surfaces we suggest that they attach primarily by polypeptide bridges anchored by lysine and/or arginine residues to GAG's of the adhesive-cell glycocalyces and GAG's of the bacterial films that coat marine substrata. Release is probably affected by the secretion of GAG's (perhaps similar to heparin) that compete with the anionic sites on the glycocalyx for the basic residues on the glue, thus the tube feet release cleanly leaving footprint films behind. The proteinaceous glue is probably contained in the small granules of the adhesive cells and the competitive GAG releasing factor is probably in the large granules of the narrow-necked cells. This model explains the data from Kerkut (1953) that show tube feet release cleanly from attached substrata and are not mechanically pulled free. It also accounts for the observation by Smith (1937) that when the ophuroid *Ophiocomina* is disturbed while adhering to the sides of aquaria it releases the bonds of all tube feet simultaneously and drops to the bottom of the tank. This model for adhesion of starfish tube feet accords with the model for podial adhesion suggested by Hermans (1983).

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THE ROLE OF EYESTALK HORMONES IN VITELLOGENESIS DURING THE BREEDING SEASON IN THE CRAB, *PARATELPHUSA HYDRODROMOUS* (HERBST)

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ABSTRACT

In *P. hydrodromous,* vitellogenesis is divided into two phases, Vitellogenesis I (V_1, subdivided into Stages 1–3) and Vitellogenesis II (V_2). The accelerated ovarian growth induced by bilateral eyestalk ablation during the first half of the breeding season apparently proceeded normally until Stage 3 of V_1. Subsequently, the yolk showed signs of impoverishment, particularly in its total protein and lipid contents. In both gross morphology and biochemistry, the ovaries of eyestalkless crabs in V_2 during the breeding season were closer to V_2 ovaries of normal crabs than to V_2 ovaries induced to develop in destalked crabs during the prebreeding season. The ovarian abnormality seen when eyestalk ablation occurs during the breeding season is not due to the unpreparedness of the oocyte population to begin vitellogenesis. In *P. hydrodromous,* the ovary, at all stages of its development except perhaps at late V_2, seems to depend on eyestalks for the normal maintenance of vitellogenesis. *P. hydrodromous* females do not spawn if eggs artificially induced to form are not normal. Influence of the eyestalk on spawning is not directly via the nervous system; an eyestalk hormone controlling oviposition, if present, is released at least one week prior to spawning.

INTRODUCTION

In many decapod crustaceans, bilateral eyestalk ablation ($-E_2$) leads to accelerated ovarian growth (Adiyodi and Adiyodi, 1970), but reports of successful spawning following such accelerated ovarian growth are few (Cheung, 1969; Hinsch, 1972). Our earlier investigations on *Paratelphusa hydrodromous* showed that the precocious ovarian growth induced by $-E_2$ during the prebreeding season (September–November), was abnormal; yolk accumulated in oocytes was biochemically impoverished and no eggs were spawned (Anilkumar and Adiyodi, 1980). Counts of avitellogenic and early vitellogenic oocytes in ovaries of *P. hydrodromous* during the prebreeding season suggested that the abnormality in ovarian physiology found in $-E_2$ females during the prebreeding season was at least in part due to the unpreparedness of a significant proportion of the oocytes to commence active vitellogenesis. This prompted us to study the effects of $-E_2$ on oviposition and the composition of yolk in crabs during the breeding season, when the oocytes are generally in a state of readiness to begin active vitellogenesis.

In the Calicut population of *P. hydrodromous,* breeding season and start of vitellogenesis begins in December. The period of vitellogenesis in *P. hydrodromous* has two phases, Vitellogenesis I (V_1) and Vitellogenesis II (V_2); the former is further subdivided into three stages (Adiyodi, 1968). During Stage 1 (V_1S_1) the ovary is whitish

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(oocyte diameter: 0.5–0.6 mm); oocytes assume a pale cream color by Stage 2 (V_1S_2) (diameter: 0.61–0.8 mm). As the ovary reaches Stage 3 (V_1S_3) the oocytes appear orange and 0.81–1.30 mm in diameter. V_2 ovaries are a deep orange with a tint of brown (oocyte diameter: 1.31–2.0 mm). The Calicut population of *P. hydrodromous* generally has a single spawning season, sometime during March/April. Eggs are carried by the female in the concavity of her broad abdomen; young are released at the monsoon onset (June). Premolt changes begin following release of the brood; molting occurs during June/July. August through November is the prebreeding season; individuals remain in intermolt and ovaries are white bands showing no signs of vitellogenic activity.

MATERIALS AND METHODS

P. hydrodromous females used in this study were collected from rice farms around the University campus and cultured in laboratory tanks as previously described (Anilkumar and Adiyodi, 1980). Crabs were reared in cement cisterns laid with wet sand at the bottom and fed ox liver *ad lib.* Acclimation to the laboratory occurred for 3–4 days prior to the experiment.

Eyestalks were removed from intermolt adult females [3.8–4.2 cm carapace width (cw)] in December. Molt stages were determined by observing the pleopods: pleopods of intermolt crabs had only a single cuticular layer; the onset of premolt was marked by the appearance of a new cuticular layer in the pleopod (after Anilkumar, 1980). For each quantitative analysis, ovaries were dissected out (by cutting open the carapace) from groups of 5 destalked animals 15, 30, and 45 days after $-E_2$. Each time, ovaries from five control crabs were also dissected out and analyzed for comparison. The Student's *t*-test analyzed the level of significance of difference between the experimentals and controls. Experimental data on crabs in prebreeding season, used for comparison in this study, have been taken from Anilkumar and Adiyodi (1980).

One hundred and forty-seven adult female crabs were sacrificed to analyze the effect of destalking on ovarian physiology and spawning during the breeding season. Further, not less than 75 freshly caught animals were used each month (throughout one full annual cycle; sample size >900) for studies on seasonal changes in the ovary.

The ovary was homogenized with 5% trichloracetic acid (TCA) to precipitate protein which, after centrifugation, was dissolved in 0.1 *N* NaOH and quantitatively estimated after Lowry *et al.* (1951). To estimate lipid, dried tissue (ovary) was subjected to Soxhlet extraction using the solvent chloroform (Vogel, 1959). Carbohydrates of the tissue were separated into ethanol (80%)-soluble oligosaccharide fraction and ethanol-insoluble polysaccharide fraction (Johnston and Davies, 1972) and estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956). Free amino acids (FAA) were extracted using 80% ethanol and estimated after Lee and Takahashi (1966). The pattern of distribution of oocytes in the ovary of both experimental and control groups was studied by making numerical counts of the yolky and yolkless oocytes; the ratio of yolky to yolkless oocytes in experimentals and controls was subjected to χ^2 test for comparison.

To evaluate the role of eyestalk hormone(s) on spawning, a series of experiments were conducted during the final phase of vitellogenesis (late V_2—March). Both eyestalks were removed from 13 adult females, out of which 6 (Group I) received regular injections of extracts of 2 eyestalks (in 0.9% saline) at a time, twice a week. A group of normal females (14) with intact eyestalks constituted the controls (Group II). Both experimentals and controls were reared in the laboratory for 11 days [during which time most of the individuals (19 out of 27) (70%)] did oviposit.

RESULTS

In December, $-E_2$ induced in crabs an initial phase of hyperphagia lasting 7–10 days; this was followed by a period of apparent normalcy in feeding pattern until 25–30 days postoperation, after which set in a phase of hypophagia lasting the duration of the experiment (45 days).

Ovarian response to $-E_2$ during the breeding season

At the beginning of the experiment in December (the first half of the breeding season of *P. hydrodromous* in Calicut), ovaries of experimentals and control crabs were in V_1S_1. $-E_2$ induced an acceleration in ovarian growth in both oocyte size (Table I) and ovarian wet weight (Table II). All experimental crabs (and controls) remained uniformly in intermolt even 45 days postoperation; a second cuticular layer was not formed in any of the experimentals showing a lack of initiation of premolt events (somatic growth).

The ovaries of experimental females which had reached V_1S_3 or V_2, 15 days postoperation (Table I), had a gross morphology comparable to that of normal females in comparable stages of ovarian development. By 45 days postoperation, the ovaries became abnormally brown and the oocytes assumed a rather flabby appearance as though they were only partially filled with yolk. The crabs become hypophagic at this time: the change in appearance of the ovary could be related, in part, to the paucity of alimentary organic resources. Chi-squared analysis, however, showed that the ratio of yolky to yolkless oocytes in the ovary of $-E_2$ females in V_2 was comparable to that of normal ovary in V_2 ($\chi^2 = 1.16$; $P > 0.2$).

A normal (control) crab required 30–45 days to reach V_1S_3 from the date of commencement of the experiment. Ovaries of destalked crabs, however, reached V_1S_3 by 15 days postoperation; in some instances the oocytes in $-E_2$ crabs were too precocious and had already reached V_2 by 15 days (Table I). Further, in experimentals, $-E_2$ led to a statistically significant precocious increase in total TCA-precipitable proteins, chloroform-extractable lipids, oligosaccharide fraction, polysaccharide fraction, and FAA content (Table II) of the ovaries, compared to controls. To evaluate whether the precociously incorporated yolk (after $-E_2$) is normal, we compared our present data with those of normal females with comparable ovarian stages (the latter from Anilkumar and Adiyodi, 1980). This (judged from the levels of organic constituents) revealed that the ovaries of $-E_2$ crabs responding precociously to the operation were normal until 15 days postoperation. The profiles of major vitelline components of $-E_2$ crabs at

TABLE I

Oocyte size and stage of the ovary after $-E_2$ during the breeding season in P. hydrodromous

Period after operation	Oocyte size in mm (O)		Stage of the ovary	
	Control (C)	Ablated ($-E_2$)	Control	Ablated
15 days (X)	0.69 ± 0.04	1.24 ± 0.44	V_1S_1 or V_1S_2	V_1S_3 or V_2
30 days (Y)	0.75 ± 0.05	1.55 ± 0.03	V_1S_1, V_1S_2, or V_1S_3	V_2
45 days (Z)	0.99 ± 0.05	1.91 ± 0.17	V_1S_3	V_2

X.O.C. < X.O. $- E_2$; $t = 2.41$ ($P < 0.05$)

Y.O.C. < Y.O. $- E_2$; $t = 13.77$
Z.O.C. < Z.O. $- E_2$; $t = 5.2$ | ($P < 0.01$)

TABLE II

Changes in ovarian wet weight and biochemical components 15, 30, and 45 days after $-E_2$ during the breeding season in P. hydrodromous *(weights shown in mg/100 g animal weight; mean ± S.E.)*

		15 days	30 days	45 days
Wet weight	Control	376.91 ± 19.80	726.34 ± 28.11	1172.08 ± 32.73
	Ablated ($-E_2$)	1630.07 ± 252.71	2998.21 ± 395.86	3513.35 ± 207.77
	$P<$	0.01	0.01	0.01
Total proteins	Control	42.35 ± 6.86	50.92 ± 7.03	257.18 ± 16.49
	Ablated ($-E_2$)	213.02 ± 31.40	370.29 ± 45.40	454.91 ± 60.09
	$P<$	0.01	0.01	0.02
Total lipids	Control	33.96 ± 5.38	52.79 ± 7.16	195.82 ± 30.42
	Ablated ($-E_2$)	225.16 ± 50.60	386.54 ± 25.30	508.38 ± 62.03
	$P<$	0.01	0.01	0.01
Oligosaccharide fraction	Control	6.08 ± 0.86	10.02 ± 2.57	11.21 ± 0.83
	Ablated ($-E_2$)	19.92 ± 3.19	22.08 ± 2.92	25.40 ± 3.87
	$P<$	0.01	0.01	0.01
Polysaccharide fraction	Control	3.68 ± 0.42	4.18 ± 0.79	4.69 ± 0.88
	Ablated ($-E_2$)	7.62 ± 1.50	10.58 ± 0.71	12.53 ± 2.08
	$P<$	0.05	0.01	0.01
Free amino acids	Control	5.16 ± 1.19	8.52 ± 1.31	10.07 ± 1.03
	Ablated ($-E_2$)	11.48 ± 1.67	21.33 ± 3.10	23.74 ± 4.88
	$P<$	0.02	0.01	0.05

P represents the level of significance of difference between the control and $-E_2$ ovaries.

V_1S_3 (15 days postoperation) were comparable (statistically) to those of a normal crab having V_1S_3 ovary. As the $-E_2$ ovaries passed from V_1S_3 to V_2 (by 30 days postoperation), however, this sense of normalcy was lost. Chief organic compounds of the yolk, particularly proteins and lipids, were impoverished in V_2 ovaries compared to normal V_2 ovaries ($P < 0.05$).

Effect of $-E_2$ on spawning

Final oocyte diameters in $-E_2$ crab ovaries often surpassed those of normal mature eggs, but none of the females, destalked in December, spawned. In contrast, normal controls maintained under identical culture conditions spawned during the normal spawning season.

In the second series of our experiments, in which adult females were destalked during the final phase of vitellogenesis (March), 66% (4 out of 6) of the Group I females, 79% (11 out of 14) of Group II females, and 57% (4 out of 7) of experimentals oviposited normally within 1 week postoperation.

DISCUSSION

If eyestalks are removed in December, oocyte growth is precipitated (Table I), the ovary gains weight, and there is a statistically significant rise in levels of different organic constituents of the ovary (Table II), all precociously in comparison to controls. This suggests that a gonad-restraining principle or principles (GIH) may be present in *P. hydrodromous* eyestalks during the breeding season. Earlier studies had shown that such a principle may also be present in eyestalks during the prebreeding season and that ovarian inactivity seen during this period is not due to ovarian refractoriness, but due to the presence of GIH in effective titres (Anilkumar and Adiyodi, 1980).

It is likely that the effects of $-E_2$, as we observed on *P. hydrodromous* ovaries, could be related to increased production of ecdysteroids (ECD). Removal of eyestalks, which are also the sources of the molt-inhibiting hormone (MIH), could lead to activation of the Y-organ and to increased secretion of ECD. A vitellogenic function for ECD has been established in Diptera among insects (Hoffmann *et al.*, 1980 for review) and is indicated in amphipods, isopods, and natantian decapods among crustaceans (Kurup and Adiyodi, 1984). Further, multiple limb autotomy (MA) in crabs accelerates the onset of molting (Skinner and Graham, 1972; Kurup and Adiyodi, 1984) or vitellogenesis (Kurup and Adiyodi, 1984), depending upon the physiological phase of the animal at the time of MA. MA causes a dramatic increase in ECD levels in the hemolymph (McCarthy and Skinner, 1977), suggesting the possible involvement of ECD component in vitellogenesis. What is puzzling, however, is that $-E_2$ did not precociously initiate premolt events in any of our crabs even 45 days after $-E_2$. The problem of involvement of the ECD component in accelerating vitellogenesis can be finally settled only when data on ECD titers in hemolymph and the degree of responsiveness of the target tissues become available for this species.

Present experiments show that eyestalks are necessary to maintain normal ovarian growth during the breeding season. Ovarian physiology is apparently normal only for about 15 days following $-E_2$. During subsequent weeks, the ovary seems to lose its ability to support a normal and properly balanced vitellogenic process in the absence of eyestalks. This is clearly borne out by the difference in wet weight during the breeding season between V_2 ovaries of $-E_2$ females and V_2 ovaries of normal females and by the decrease in quantity of yolk (reflected in wet weight of the ovary) and in quantities of the chief organic constituents of yolk. In V_2 ovaries of $-E_2$ females (not controls), certain flabby oocytes often larger than normal mature eggs, but incompletely filled with yolk, are present. This indicates that accumulation of yolk within oocytes following $-E_2$ could be abnormal at least in one population of oocytes.

Comparison between V_2 ovaries of normal crabs and $-E_2$ crabs revealed that major organic components of yolk whose synthesis or accumulation in oocytes is most affected by $-E_2$ are proteins and lipids. The fall in organic reserves of the ovary, noticed in crabs 30 days postoperation, is unlikely to be related to lack of feeding, for $-E_2$ crabs do not cease feeding until 25–30 days postoperation. We do not rule out the possibility that metabolic derangements resulting from lack of food may, however, be responsible, at least in part, to the impoverished nature of the $-E_2$ crab ovary, 45 days postoperation. $-E_2$ may gradually render the oocyte synthetic machinery inefficient or hinder uptake of vitellogenin and utilization of lipid from blood by the ovary.

Abnormal ovarian physiology, seen during the breeding season in $-E_2$ crabs, cannot be explained as the consequence of a hasty initiation of the vitellogenic process in an otherwise unprepared ovary, for the operation was conducted at a time when the ovary was ready to begin normal vitellogenesis. The eyestalk is, therefore, *sensu stricto* not inhibitory, but possibly is a seat of some 'restraining factor', which seems to be essential to the normal progression of vitellogenesis. The question arises whether the precocious accumulation of yolk reserves and subsequent malfunction of the ovary consequent upon $-E_2$ is due to the absence in circulation of GIH and/or to some metabolic principles contained in eyestalks. As ovary is the target tissue involved, GIH appears to be the most likely candidate which restrains oocyte growth, but whose presence is, nevertheless, necessary for proper ovary function. It may be argued that the term GIH denotes not a single hormone, but a group of factors some of which may be necessary for normal differentiation of the oocytes and others to regulate the many metabolic processes that help maintain normal vitellogenesis. Eyestalks in decapod crustaceans contain factors influencing the metabolism of carbohydrates (Chang and O'Connor, 1983), lipids (Chang and O'Connor, 1983), and nitrogenous substances (Claybrook, 1983), but to what extent they specifically influence ovarian metabolism is unknown.

A comparison of the biochemical composition of V_2 ovaries of $-E_2$ females whose eyestalks were ablated during the prebreeding season (the data from Anilkumar and Adiyodi, 1980) with V_2 ovaries of $-E_2$ females destalked during the breeding season reveals that, despite the differences discussed above, the latter are closer to ovaries from intact female in V_2. Further, our previous analyses (Anilkumar and Adiyodi, 1980) revealed that during the prebreeding season ovarian abnormality resulting from $-E_2$ manifests itself from 15 days postoperation, when the ovaries are in V_1S_3, whereas during the breeding season (present study) such abnormality becomes evident only over a longer period. We do not know whether the difference in response of crabs destalked during the prebreeding season is directly or indirectly related to hemolymph protein levels (Anilkumar, 1980). One basic difference between crabs destalked during prebreeding or breeding seasons is the ratio of yolky to yolkless oocytes in ovaries. Experimental crabs destalked at the beginning of the breeding season have oocytes in V_1S_1, whereas ovaries of intact crabs at the beginning of the prebreeding season have not reached V_1S_1, and most of the oocytes have diameter less than 0.5–0.6 mm (Pillai and Adiyodi, unpub.). The uneven acceleration of vitellogenesis, apparent in ovaries of $-E_2$ females during the prebreeding season (Anilkumar and Adiyodi, 1980), is thus primarily a function of the age of oocytes at the time of eyestalk removal. Pillai and Adiyodi (unpub.) further observed that the oocytes of *P. hydrodromous* occupy their definitive positions in the ovary following the postspawning revival of activity of the germarium, but have to grow considerably (perhaps along with some cytological differentiation), before acquiring the characteristics of oocytes in V_1S_1. This was further substantiated in our laboratory by another series of experiments (Kurup and Adiyodi, in prep.). In *P. hydrodromous*, destalked during the postspawning period, the ovary responded by accelerated growth, but the oocytes did not grow beyond V_1S_2 or V_1S_3. Again, the pattern of oocyte response was uneven, only a few oocytes reached V_1S_2 or V_1S_3 (Kurup and Adiyodi, in prep.).

The ovary of *P. hydrodromous* seems to require eyestalk factors (possibly GIH) at all stages of its development except perhaps at the final phase of vitellogenesis (late V_2). When destalking was conducted during the postspawning season, only a few oocytes responded to $-E_2$ and even they did not grow beyond V_1S_2 or V_1S_3 (Kurup and Adiyodi, in prep.); few oocytes reached V_2 under $-E_2$ during the prebreeding season (Anilkumar and Adiyodi, 1980). Our present study reveals that during the breeding season the oocytes of $-E_2$ females reached V_2 much sooner, *i.e.*, by 30 days postoperation; at this time, however, ovarian biochemical impoverishment occurred, and the operated individuals could not successfully complete vitellogenesis. When eyestalk ablation was conducted during the final phase of vitellogenesis (March), however, most of the individuals could successfully complete vitellogenesis and spawning; the dependence on eyestalk (for maintenance of normalcy of ovarian development) apparently stops once oocytes reach late V_2.

Mating occurs in *P. hydrodromous* during late June–early August, between a soft postmolt female and a hard intermolt male. During the first breeding season following pubertal molt, young female *P. hydrodromous* do not commence vitellogenesis if mating does not take place, probably because there is no chance of fertilization (Krishnakumar and Adiyodi, in prep.). Successful mating is no guarantee that spawning will take place; spawning will not occur if ovarian normalcy is disturbed, for instance, by $-E_2$. Eyestalk influence, if any, on spawning is not via the nervous system; ablation of eyestalks during the final phase of vitellogenesis did not inhibit oviposition. MA, carried out during the reproductive phase, accelerated ovarian growth in *P. hydrodromous* but such females failed to oviposit (Kurup and Adiyodi, 1984). The same operation when conducted during the final phase of vitellogenesis (*i.e.*, 12–20 days prior to oviposition) did not induce any perceptible acceleration in ovarian growth or interfere with the spawning process (Kurup and Adiyodi, 1984). The results reveal that a block to the release of eyestalk principle(s) either by $-E_2$ or MA in turn blocks spawning except perhaps in the final phase of vitellogenesis. We do not know whether the eyestalk is the source of a separate oviposition-inducing hormone as suggested by Bomirski and Klek (1974) in *Rithropanopeus harrisii.* Regardless, during our destalking experiments conducted in March, two of the eyestalkless animals oviposited only on the 7th day postoperation. We therefore suggest that if the eyestalks of *P. hydrodromous* are seats of oviposition-inducing hormone (as suggested in *R. harrisii*), the hormone is released at least one week prior to spawning.

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INHIBITION OF SEA URCHIN EGG MITOSIS BY RETINOIC ACID AFTER NEAR-UV LIGHT EXPOSURE

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ABSTRACT

Not only can chemicals enhance the cellular damage of near-UV light (photosensitization), but we now report that previous exposure to near-UV light can enhance chemically induced cellular damage. Fertilized sea urchin eggs were used to test all trans retinol, retinal, and retinoic acid as near-UV photosensitizers. Inhibition of cell division was not observed when fertilized eggs were exposed to the light in the presence of retinol or retinal. Retinoic acid and UV-exposure together partially prevented cell division. When fertilized or unfertilized eggs were pre-exposed to near-UV light and then 10^{-4} *M* retinoic acid added to the cultures, cell division was totally prevented. The inhibition of cell division was not observed due to any combination of UV exposure and/or addition of retinol or retinal. This work shows that near-UV light can sensitize the direct retinoic acid-mediated prevention of sea urchin egg division.

INTRODUCTION

Chemicals can enhance the cellular photodamage of near-UV light (photosensitization). We now report that near-UV light can enhance chemical damage to cells. Several previous reports (Epstein, 1977; Forbes *et al.,* 1979, 1980) have shown that after exposure of mice to non-toxic levels of UV-B radiation, the feeding of non-toxic levels of all-*trans*-retinoic acid (RA) altered the growth pattern of their skin cells. They developed tumors much more readily than the UV-irradiated or the RA-fed animals not exposed to this light.

We have been concerned that retinoids may be involved in the UV-photosensitization of ocular tissues via the formation of toxic photoproducts. We found, however, that this hypothesis was not upheld by experimentation, since neither retinol, retinal, nor retinoic acid, after they were exposed to long wavelength UV-light (365 nm), adversely influenced the survival or mitosis of sea urchin eggs. While yellow solutions of vitamin A become irreversibly bleached within a few hours by exposure to near-UV light (365 nm ± 20 nm; $50W/M^2$), the bleached products of vitamin A did not kill or stop the growth of dinoflagellates. Subsequently, experiments were undertaken using fertilized sea urchin eggs as test systems and all-*trans* retinol, retinal, and retinoic acids as potential photosensitizers. No photosensitization effects were observed. However, when eggs were exposed to near-UV light (as above) only the addition of the retinoid retinoic acid to the cultures prevented the division of sea urchin eggs.

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MATERIALS AND METHODS

Eggs and sperm of sea urchins (*Arbacia punctulata* and *Lytechinus variegatus*) were obtained by injecting the animals with 0.5 ml of 3 *M* KCl. Millipore-filtered seawater was used for all collections and washings. Eggs were washed three times before use. Sperm samples were suspended in millipore-filtered seawater and diluted 20 fold before addition to egg suspensions for fertilization.

All-trans retinol, retinal, and retinoic acid (Sigma Chemical Co.) were dissolved in 100% methanol at 5 m*M*. Methanol solutions of the retinoids (0.1 ml) were added to seawater containing eggs (5 ml) to give a final concentration of 0.1 m*M*. The addition of methanol alone did not influence the fertilization or cell division processes of near-UV irradiated (as below) or unirradiated eggs.

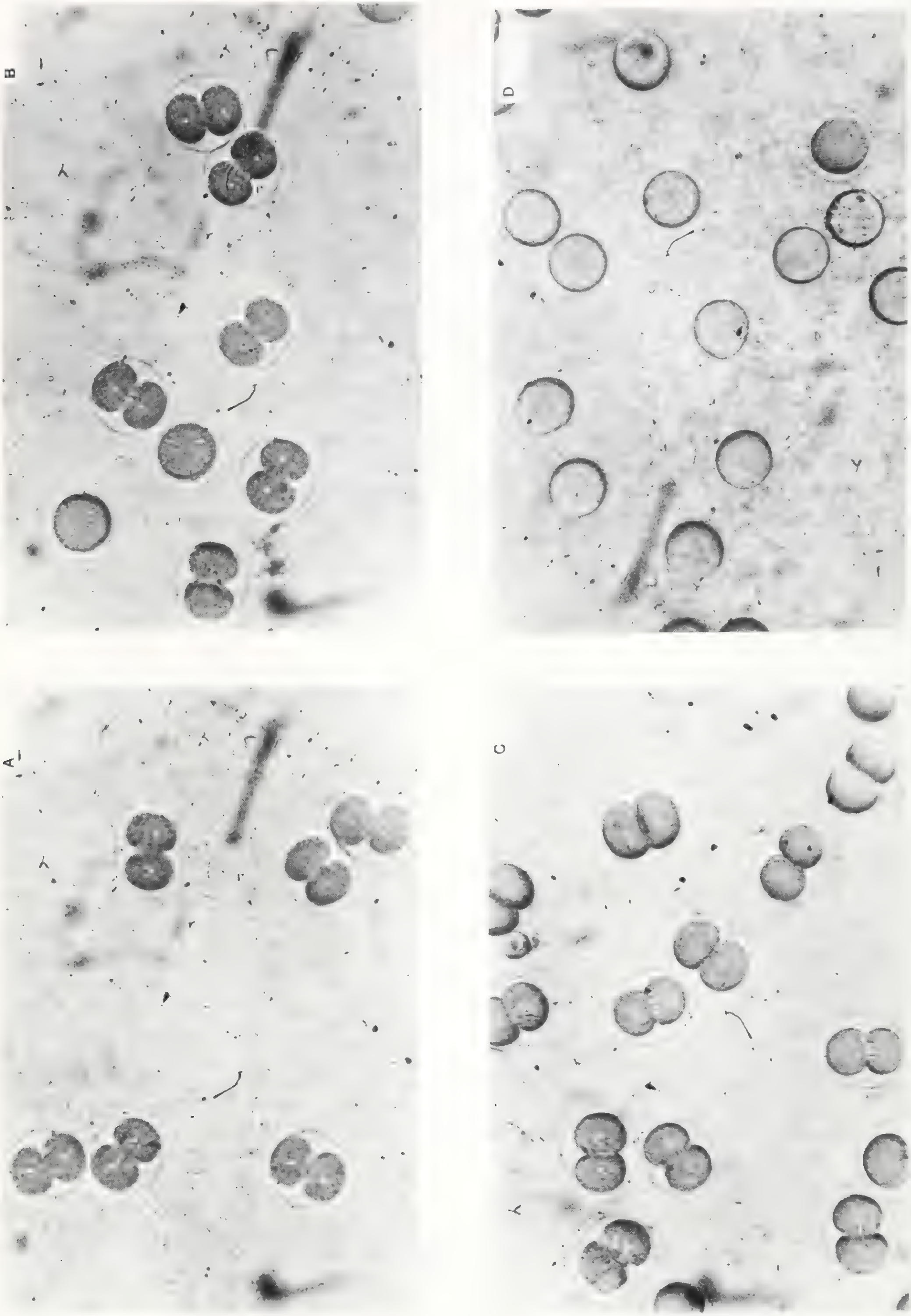
The lamp used to irradiate the eggs and the retinoic acid solutions was a long-wavelength emitting UV grid lamp 9PCQ 008L; Ultraviolet Products, Inc. long-wave UV meter (J 221) was $20W/M^2$ at a distance of 6 inches. The position of the egg suspension and the time of exposure of the eggs were varied from 2 to 24 h, providing a range of exposures between 1.4×10^5 and $1 \times 6\ 10^6\ J/M^2$.

Experiments were carried out as in previous studies (Zigman and Hare, 1976; Zigman and Gilman, 1980). Eggs were obtained from several sea urchins. Five-ml portions of the stock suspensions were poured into 10-ml pyrex beakers and kept under the near-UV lamp, as above, with a 2-mm pyrex watch glass covering them. The suspensions were irradiated for 2, 3, 4, and 24 h in a water bath at 20°C. At the appropriate times, the eggs were removed from the UV chambers and sperm suspen-

TABLE I

Effects of retinoic acid (RA) and n-UV light on sea urchin egg development

Treatment	First Division		One day embryonic development
	Time post-fertilization	% Dividing	
1. None	60 min	90	Normal
2. Plus MeOH at 20 μl/ml	60 min	90	Normal
3. Plus RA at 10^{-4} *M*	70 min	80	Slightly delayed
4. Exposed to N-UV light through pyrex glass at $20W/M^2$ for 24 h	90 min	89	Slightly delayed
5. Plus RA (10^{-4} *M*) and exposed to N-UV as above for 1 h simultaneously	70 min	80	Slightly delayed
6. Same as 5. for 2 h	85 min	50	To 64–128 cells
7. Same as 5. for 3 h	None	None	None
8. Exposed to N-UV light (as above) for 24 h; than MeOH added-(20 μl/ml)	90 min	80	Slightly delayed
9. Plus RA (10^{-4} *M*) after N-UV exposure (as above) for: 2 h	65 min	80	Mostly normal
3 h	70 min	50	To 64 or 128 cells
4 h	90 min	2	None
24 h	None	None	None


B
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A
C

sions were added to fertilize them. Fertilization membranes rose in 3 min, at which time the retinoid to be tested was added. A binocular compound microscope was used to observe the developmental events, and a Pentax Spotmatic 35 mm camera, attached through an adaptor, was used to photograph the eggs. In accompanying studies, eggs were fertilized and observed after UV exposure only, after retinoid exposure only, and after prior exposure of the eggs to retinoids and then to the near-UV light. None of the retinoids previously exposed to UV and added to sea urchin eggs influenced their division.

In another experiment, 2 μCi of 3H-thymidine (20 m Ci per m*M*) was added to the 5 ml of seawater in dishes containing freshly fertilized sea urchin eggs. At times from 20 to 100 minutes, 5 ml of 10% TCA was added to the dish to terminate incorporation and the cells were harvested and dissolved in 0.1 *N* NaOH. Aliquots of the precipitated material from each plate were counted in a Packard Tri-carb liquid scintillation counter. The categories studied were controls, UV only, 10^{-4} *M* retinoic acid only, and eggs UV-irradiated prior to the addition of retinoic acid.

RESULTS

Table I summarizes the findings of our experiments. We found no photosensitizing action for UV-light exposure of any of the retinoids, and none of the retinoids were photo-oxidized to toxic products as tested against sea urchin egg mitotic activity. Methanol solutions of retinol had no influence on sea urchin mitosis whether in natural yellow form or bleached by near-UV light for four hours. Retinoic acid alone at 0.1 m*M* had little effect on sea urchin egg division or subsequent growth. When eggs were exposed to both retinoic acid and near-UV radiation together, cell division was markedly inhibited (see Table I). A dramatic total inhibition of mitosis also resulted when unfertilized sea urchin eggs were exposed to near-UV light for a period as short as 3 h, followed by addition of 0.1 *M* retinoic acid at 3 min postfertilization. Similar results were obtained when the retinoic acid was added to irradiated eggs before fertilization.

The near-UV enhanced prevention of sea urchin egg mitosis by retinoic acid is clearly shown in Figure 1, which shows the morphologic appearances of eggs treated as stated above. There is no influence of any of these treatments on the fertilization process as shown by the prompt appearance of the fertilization membranes. Only when the eggs were exposed to both factors or were pre-exposed to near-UV light and then retinoic acid was added to their suspensions (in this case after fertilization) was cell division prevented.

Figure 2 illustrates a time course of 3H-Tdr incorporation into the DNA of the developing sea urchin eggs. UV exposure of the eggs had little influence on Tdr incorporation. Retinoic acid alone inhibited Tdr incorporation after 40 min. Retinoic acid added to the system after UV-irradiation led to cessation of DNA synthesis. The degree of inhibition was much greater than for retinoic acid alone up to 40 min, but subsequently no further incorporation occurred.

The amount of inhibition of DNA synthesis due to the toxicity of retinoic acid alone was insufficient to totally stop mitosis, whereas the same concentration of retinoic acid added to the eggs after pre-UV irradiation totally prevented mitosis.

FIGURE 1. Sea urchin (*Lytechinus variegatis*) eggs at one hour postfertilization with the following treatments: (A) control; (B) with 10^{-4} *M* RA added; (C) after exposure to near-UV light for four hours before fertilization; (D) with 10^{-4} *M* RA added after pre-exposure to near-UV light for four hours.

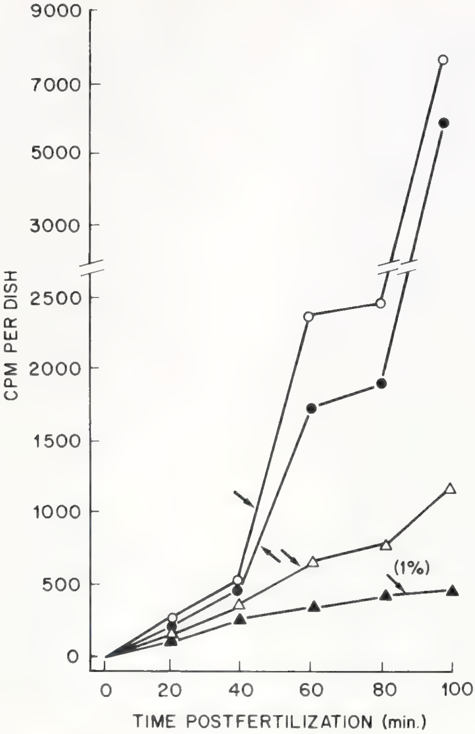

FIGURE 2. Time course of incorporation of ^{3}H-Tdr into the DNA of fertilized sea urchin eggs. ↗ = first division occurs, 1% = percent of cells dividing; open circles = control; closed circles = irradiated with no additives; open triangles = retinoic acid added, but no irradiation; closed triangles = 8 h UV pre-irradiation, then retinoic acid added. Conditions of the experiment are stated in the Materials and Methods section.

DISCUSSION

This study demonstrates that near-UV light at irradiances not greater than those found in the natural environment or derived from many artificial lamps, can sensitize cells so that non-toxic biochemicals become toxic to them. Because mitosis is a complicated process, it is not possible to suggest a specific mechanism of action for the near-UV chemosensitization of sea urchin eggs by retinoic acid. Further studies are needed.

Whatever the mechanism is, it appears to be related to an early inhibition of DNA synthesis which interferes with cell division and further development. While retinoic acid alone adversely influences DNA synthesis, the effect occurs too late or at too minor a degree to stop mitosis. On the other hand, pre-UV-exposed eggs do suffer sufficient DNA synthesis inhibition to stop mitosis.

The enhancement of skin tumor development due to pre-exposure to near-UV radiation and then retinoic acid application was shown by others (Epstein, 1977; Forbes *et al.*, 1979) to enhance abnormal development of cells. Thus, we report a phenomenon not generally known or understood, but which seems to have a great significance in the study of near-UV light effects on many types of cells studied in biology and medicine.

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